CHARACTERIZING BONE LOSS IN AN OVARY-INTACT MURINE MODEL OF MENOPAUSE

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STATEMENT BY AUTHOR

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ABSTRACT

The industrial chemical 4-vinylcyclohexene diepoxide (VCD) induces gradual ovarian failure in mice by accelerating the natural process of ovarian follicle atresia. Following follicle depletion, residual ovarian tissue in VCD-treated rodents secretes bone-protective androgens. It is hypothesized that VCD will not affect bone prior to ovarian function changes in Sprague Dawley (SD) rats. Young (1 month) and mature (3 month) SD female rats were administered VCD (160 mg/kg, ip) or vehicle (DMSO, 1.25 μL/g, ip) for 25 days. Following treatment the rats were monitored daily for ovarian function, with histological assessment of ovarian follicles post-sacrifice. Bone mineral density (BMD) and static bone histomorphometric indices were analyzed. Immediately post-treatment, all rats displayed regular estrous cycles. However, young SD rats demonstrated significantly decreased BMD in the total femur, distal femur, total tibia, and proximal tibia immediately post-VCD. Mature VCD-treated rats demonstrated significantly decreased BMD in the total and proximal tibia. Histomorphometry of the proximal tibia in young VCD rats supported these findings. Contrary to our hypothesis, VCD appears to have bone effects in SD rats independent of changes in ovarian function. The deleterious effects of VCD on bone in SD rats may compromise the utility of VCD in this animal for bone research.
STATEMENT OF PURPOSE

The industrial chemical 4-vinylcyclohexene diepoxide (VCD) induces gradual ovarian failure in mice by accelerating the natural process of ovarian follicle atresia. Following follicle depletion (~5 months post-VCD), residual ovarian tissue in VCD-treated rodents continues to secrete bone-protective androgens. Previous research has demonstrated that bone loss in the vertebrae, distal femur, and femoral midshaft of C57BL/6Hsd mice administered VCD (160 mg/kg; i.p.) occurs later and to a lesser extent than in ovariectomized (OVX) mice. However, the effects of VCD on bone have not been characterized in rats. It is hypothesized that, analogous to what occurs in mice, VCD will not affect bone prior to ovarian function changes in Sprague Dawley (SD) rats. The purpose of this thesis was to test the hypothesis that 4-vinyl-1-cyclohexene diepoxide (VCD) has no negative effects on the bone of Sprague Dawley rats, immediately post-VCD administration.

The first aim of the study was to establish the ovotoxicity of VCD in Sprague Dawley rats. The second aim of the study was to establish the acute in vivo effects of VCD administration on Sprague Dawley rats that are either growing or skeletally mature at the onset of dosing. Based on the outcomes of these two aims, the third aim of the study was to establish the in vitro effects of VCD on osteoclast differentiation (osteoclastogenesis) using two different cell culture systems – an osteoclast precursor-only model (RAW 264.7 cells) and a mixed bone marrow cell culture.
STATEMENT OF RELEVANCE

The U.S. Food and Drug Administration (FDA) mandates that the safety and efficacy of drugs developed to prevent or treat post-menopausal osteoporosis should be demonstrated in an ovariectomized rat model. These OVX rats need to demonstrate cancellous bone loss. In addition, the femur, tibia, and lumbar vertebrae of these rats must be monitored via histomorphometry, bone density analysis, and biomechanical testing.\[107]\n
In their 1994 “Guidelines for preclinical and clinical evaluation of agents used in the prevention or treatment of postmenopausal osteoporosis,” the FDA stated: “At the present time, an experimental model that precisely mimics the pathophysiology of postmenopausal osteoporosis is unavailable. Although several risk factors for osteoporosis have been identified, there is a predominant association with estrogen-deficiency. Hence, ovariectomized animals are the preferred animal models to provide insight into the clinical outcome of an anti-osteoporotic drug”.\[107]\n
However, the VCD model of menopause was developed as a more realistic model of human menopause than traditional OVX models. In the murine VCD model, a perimenopausal transition occurs that mimics both the hormonal and cyclicity changes observed in humans.\[23]\nTherefore, it is important to verify that the VCD model does not directly affect cancellous bone, if it is to supplement studies involving the FDA’s mandated OVX model for monitoring osteoporotic bone loss.

Only two experimental studies have analyzed the effect of VCD in Sprague Dawley (SD) rats. Neither of these studies examined the effects of VCD on bone.\[50, 59]\ Because SD rats are the most commonly utilized strain of outbred rat in research laboratories that employ animal models, it is important to examine the effects of VCD on bone in these animals.\[106]\ If bone
changes do not occur immediately following VCD treatment in SD rats, it can be assumed that VCD is not directly toxic to bone. Subsequently, a protocol can be developed for a VCD-treated model of menopause in SD rats that can be utilized in studies of post-menopausal bone loss.
OVARY / OVARIAN FUNCTION

The Rat Estrous Cycle

The word “estrus” is derived from the Greek word “oïstros,” meaning “gadfly, sting, or frenzy.” Originally, this term described the “special period of sexual desire of the female.” Other words were developed to describe the specific reproductive stages that occur in mammals that breed seasonally. However, these terms were eventually applied to the various stages of all mammals displaying estrous cycles.\[^{81}\]

The estrous cycle in rats is characterized by four phases: proestrus, estrus, metestrus, and diestrus. The entire cycle usually occurs over four consecutive days. However, the characteristics of the estrous cycle can vary greatly between individual rats and the length of rat estrous cycles can change in response to multiple social and environmental factors. For example, female rats housed alone or with other females will cease cycling earlier as compared to female rats housed with males. In contrast, other factors such as exposure to light, do not affect either the frequency or regulation of the rat estrous cycle.\[^{76}\]

Rats begin estrous cycling as soon as the vaginal orifice opens. This occurs approximately 32-36 days after birth. The estrous cycles will continue until approximately 10-12 months of age.\[^{86}\] Laboratory rats ovulate spontaneously, ovulate multiple times in a year, and continuously display ovarian cycles throughout the year. The onset of ovulation is not dependent on nervous stimulation and occurs every 4-5 days. On average, proestrus lasts for 12-14 hours, estrus lasts for 25-27 hours, metestrus lasts for 6-8 hours, and diestrus lasts for 55-57 hours.\[^{81}\]

Ovarian Follicles in Rats
An ovarian follicle consists of a follicular basement membrane that surrounds somatic cells and an oocyte. Ovarian follicles first develop during the embryonic stage of development. Initially, germ cells migrate to the gonad and begin to undergo mitosis. After entering meiosis, all of the existing germ cells become arrested in the diplotene stage of the first meiotic prophase. At this point, the germ cells become surrounded by somatic cells known as “granulosa cells” and are known as “primordial follicles.” These granulosa cells stem from embryonic stroma.

The follicles present in the ovary can be generally classified into four groups: small follicles that are quiescent, small follicles entering the pool of growing follicles, large preantral follicles, and large antral preovulatory follicles. A few follicles will begin to grow during each day of the ovarian cycle, until they either ovulate or undergo atresia.

A rat primordial follicle typically displays approximately four granulosa cells when viewed on a slide in cross-section. Primordial follicles are those follicles that display a single layer of simple squamous epithelium surrounding the oocyte. Primordial follicles progress to primary follicles when the simple squamous epithelium enlarges to form low columnar epithelium. This occurs in the neonate. The simple columnar epithelium will multiply, forming in turn a secondary follicle with two layers of columnar epithelium followed by an antral follicle with stratified cuboidal follicular epithelium. The antral follicle, also known as a Graafian follicle or tertiary follicle, contains a single large fluid-filled intracellular space known as the antrum. During the progression between the secondary and antral follicles, the oocyte moves away from the center of the follicle and forms a hillock known as the cumulus oophorus. At this point the granulosa cells surrounding the oocyte are termed the corona radiata. Prior to ovulation, the fluid in the antrum, known as the liquor folliculi, increases in volume. During the development of the secondary follicle, the theca cells form from the stroma. The theca interna
and theca externa cells form an outer sheath that encapsulates the follicle. The theca interna cells appear polygonal with a vacuolated cytoplasm and a vesicular nuclei. The theca externa cells contain contractile tissue, which will later aid in the process of ovulation. The theca cells lie in a network of reticular and fibrous fibers that contain capillaries and lymphatics.[81]

In fully mature follicles, granulosa cells display LH receptors, FSH receptors, somatomedin C receptors, insulinlike growth factor-1 (IGF-1) receptors, GnRH binding sites, and α-adrenergic binding sites. Granulosa cells will also contain enzymes such as aromatase and cholesterol side-chain-cleavage cytochrome P-450. Fully developed granulosa cells will also express genes for inhibin, IGF-1, tubulin, proenkephalin, and tissue-type plasminogen activator. Secretory products of mature granulosa cells include a heat-shock protein (hsp90), anti-Mullerian hormone, and prostaglandin E₂ that is stimulated for release by LH. FSH triggers the maturation of granulosa cells in the late stages of follicular development, resulting in the appearance of LH receptors and the enzyme aromatase, and the expression of plasminogen activator, IGF-1, and the gene for inhibin. The mature theca cells synthesize androgens, rennin, relaxin, prostaglandins, and angiogenic factors that simulate proliferation of endothelial cells.[87]

**Corpora Lutea**

Corpora lutea are derived from granulosa and theca cells of the prevoluntary follicle. Corpora lutea are formed in the diestrus phase, but do not begin to degenerate until the metestrus stage of the next ovarian cycle. The surge in prolactin secretion that occurs during mid-proestrus acts to maintain the progesterone-secreting corpus luteum. By diestrus of the next cycle, the corpus luteum has regressed. Prior to regression, the corpus luteum will secrete low levels of progesterone. Ovulation occurs in the diestrus of the following cycle as the secretion of luteal
progesterone during the “luteal phase” declines. More than three corpora lutea, the remnants of past ovulatory cycles, may be present at any one time in the ovary. This is because corpora lutea can persist for 12-14 days.[81]

Fig. 1.1 Diagram depicting the stages of folliculogenesis.[75]

**Meiosis I**

When germ cells cease dividing and begin the process of meiosis, they are considered oocytes. Oocytes begin to progress through the prophase stage of meiosis, which is subdivided into leptotene, zygotene, pachytene, and diplotene. The meiotic process is arrested in the diplotene stage. At the same time, the cells that will develop into the granulosa cells stop dividing and become quiescent. At this point, these pre-granulosa cells appear crescent shaped. Meiosis and cell proliferation will not continue until the primordial follicle begins growth, months or even years later.[87] Meiosis will be re-initiated following a surge in LH secretion.[76]
**Resumption of Meiosis**

Quiescent primordial follicles can resume growth immediately after they are formed, and can continue to be reactivated until the end of the reproductive life of the rat. Some follicles begin to grow at all time points after the primordial follicles have formed, resulting in a steady stream of growing follicles.\(^{[87]}\)

When the germ cells do resume meiosis, the granulosa cells will proliferate, while the oocyte will undergo an increase in size. When meiosis resumes in these follicles, the granulosa cells will generally undergo around ten series of mitotic divisions. These divisions occur at a more rapid pace as follicular growth proceeds. The granulosa cells will only become mature during the last round of cell division, when they will develop the ability to synthesize steroids, protein hormones, and LH receptors. Interaction between LH and the LH receptor on the granulosa cells will trigger the process of ovulation.\(^{[78]}\) The final phases of meiosis will only occur after fertilization.\(^{[76]}\)

**Folliculogenesis**

The pool of dormant primordial follicles in a female mammal is finite in number.\(^{[78]}\) These follicles appear to be randomly distributed throughout the ovary.\(^{[84]}\) Ovaries of mature rats contain large numbers of quiescent primordial follicles and slightly lower numbers of growing follicles. Each follicle of the ovary follows the same developmental pathway, but begins this process at different times during the life of the rat. Therefore, the rate at which individual follicles progress through the different stages of folliculogenesis may vary. In addition, the rate of folliculogenesis may vary between individual rats.\(^{[87]}\)
When growth of the primordial follicles resumes, the squamous granulosa cells are the first cells to recommence proliferation. As the granulosa cells proliferate, they develop a cuboidal shape. The volumes of the oocyte nucleus and cytoplasm increase sharply. The oocyte will reach full development early on during folliculogenesis, while the granulosa cells will continue to proliferate. The four granulosa cells present in the primordial follicles will double during each generation of growth. Therefore a fully developed preovulatory follicle that contains 2000-2500 granulosa cells in cross section will have taken 10 generations to develop. The zona pellucida will also begin to develop soon after the primordial follicle resumes growth. The proteins present in the zona pellucida appear to be secreted by the oocyte. Very small follicles display theca cells that will continue to grow along with the follicle. Arterioles form to supply follicles with their own blood supply by the time they have reached 80-100 μm in diameter. Each primordial follicle is surrounded by a basement membrane. The basement membrane surrounding a primordial follicle of 25 μm in diameter would need to cover a surface area of 1965 μm², while a fully grown preovulatory follicle of 500 μm in diameter would require a basement membrane that covers a surface area of 785,600 μm². Therefore, during folliculogenesis the basement membrane surrounding the follicle must increase over 400-fold in size. Eventually an antrum will form in the follicle. Fluid-filled spaces will appear in between granulosa cells. These dispersed areas will eventually coalesce into a single fluid-filled antral cavity. This fluid is formed as a result of the filtration of blood in the thecal layers through a molecular sieve. Substances larger than a molecular weight (MW) of 850,000 will not be able to enter the antral fluid, while only 50% of molecules of MW 250,000 will be filtered into the antral fluid. The formation of the antrum marks the end of folliculogenesis. Cell proliferation will
cease and the granulosa cells closest to the basement membrane will begin to assume a columnar appearance.\textsuperscript{[87]}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig12}
\caption{Flow diagram depicting folliculogenesis.\textsuperscript{[78]}}
\end{figure}

\textbf{Atresia}

Four separate periods of germ cell degeneration occur in the rat ovary, beginning with the onset of germ cell meiosis. Approximately 18.5 days after conception, rat ovaries contain 75,000 germ cells. However, by 2 days after birth, this number has been reduced to 27,000.\textsuperscript{[87]} It is
estimated that by 20 days after birth the total number of oocytes in the ovary has decreased from
35,000 to 11,000. This is due to the process of atresia.[81]

Atresia continues at much lower rates throughout the life of the rat. During each ovarian
cycle, atresia occurs primarily in secondary and antral follicles.[81] Unless a follicle continues to
undergo growth and develop it will become atretic and degenerate.[76] The mechanism by which
some follicles ovulate while other follicles undergo atresia has not yet been determined. Atresia
in antral cells is first observed in the epithelial cells lining the antrum. In secondary cells, atresia
is first noted in the cells directly external to the corona radiata. Atresia begins with pyknosis of
the granulosa cells, wherein the nucleus shrinks and the chromatin mass condenses.
Karyorrhexis may also occur, wherein the nucleus of the granulosa cells ruptures, releasing
chromatin that disintegrates into granules that are extruded from the cell. In antral follicles,
pyknotic cells and chromatin fragments slough off into the antral fluid and can be observed
under a microscope. As the cumulus oophorus shrinks, the ovum will eventually enter the antral
fluid. The zona pellucida will then degenerate, followed by degeneration of the oocyte. The
granulosa cells will eventually degenerate completely. The theca interna cells remain as
interstitial masses that will eventually degenerate into small clusters of cells in the medulla of the
ovary.[81]

Primordial follicles become depleted as follicles grow and as follicles undergo atresia. A
majority of follicles will never fully mature, but will undergo atresia. Atresia initially presents as
a few necrotic granulosa cells. Within 24 hours, multiple degenerating granulosa cells are
apparent. Within a few days, only remnants of the granulosa cells and the oocyte remain. Theca
cells hypertrophy during atresia, resulting in secondary interstitial tissue that secretes androgens
in the ovary. Increased FSH levels during estrus are required to prevent atresia in follicles that
have reached the 8th to 9th generation of development and are approximately 300-400 μm in diameter. This is the period during which most follicles undergo atresia. In immature rats, that do not display increases in FSH levels, all follicles that grow to 300-400 μm in diameter undergo atresia.[87] In experimental studies, the administration of gonadotropin from a pregnant rat during the time when FSH levels would fall (on the morning of estrus) reduces the atresia of large ovarian follicles.[76]

**Follicle Size Changes during the Estrous Cycle**

Between the time a follicle begins to grow and the time of ovulation, 19 days will have elapsed. Follicles in the large preantral stage that do not undergo atresia are considered “committed” to the pool of follicles that may ovulate during the next proestrus phase of the ovarian cycle. Maturation of the follicles must coincide with the surge in gonadotropic hormones for ovulation to occur. During late proestrus, preovulatory antral follicles are approximately 500-750 μm in size. During the post-ovulation metestrus, only follicles 390-500 μm in size are present in the ovary. During diestrus, follicles larger than 500 μm are present. Therefore, it would appear that follicles begin their growth in the proestrus phase and those follicles that reach 390-500 μm in size by late estrus/early metestrus are likely the follicles that progress through diestrus and are ultimately ovulated.[81]

During estrus and metestrus, only small follicles (390-500 μm in diameter) are apparent in the ovary. Follicle size continues to increase throughout the estrous cycle, with follicle diameter reaching a peak during proestrus. Since few small follicles are seen after metestrus, it is likely that the small follicles observed in estrus and metestrus grow and mature into larger follicles. Therefore it would appear that, on average, 3 days elapse between follicular growth
from 390 μm in diameter to mature follicle size. Follicles that are smaller than 390 μm in diameter appear on all days of the estrous cycle in the rat; however, large preovulatory follicles are only apparent during late diestrus and proestrus. This would suggest that the ovary can only respond to the surge in LH for a limited period of time during the estrous cycle. Follicular atresia was most apparent in follicles 200-400 μm in diameter. This suggests that a signal is necessary to prevent the normal atretic process that occurs in follicles of this size, in order for ovulation to proceed. It has been hypothesized that the surge in FSH is responsible for preventing atresia in follicles 200-400 μm in diameter.\[84\]

![Diagram of follicle growth stages](image)

**Fig. 1.3** Diagram depicting the size of follicles during folliculogenesis.\[52\]

**Time Course of Folliculogenesis**

Follicles will undergo folliculogenesis at varying timepoints during the reproductive lifespan, resulting in a constant stream of growing follicles in the female ovary following reproductive maturation.\[78\] Follicle growth will only cease when the follicle undergoes ovulation or atresia. The number of follicles that can be observed at any given stage of growth depends on the rate of growth initiation, the rate of follicle growth, and the rate of follicular
Overall, a large number of follicles will be observed in the primordial stage, while fewer numbers of large follicles will be observed. This is compounded by the fact that follicular growth in large preovulatory follicles occurs at a much more rapid pace as compared to early primordial follicles. Individual follicles can vary in the period of time required for folliculogenesis.[78]

The mechanism by which an oocyte is induced to undergo maturation during proestrus has not been determined. Until proestrus, the oocyte remains arrested in the dictyate stage of meiosis I. A preovulatory surge of LH controls the completion of the meiotic division in the oocyte. Meiosis I resumes during proestrus, approximately 2-3 hours after the release of LH and approximately 7-9 hours before ovulation occurs. The oocyte becomes haploid (the chromosomal number is reduced from 42 to 21) when the first polar body is extruded during telophase I of meiosis I. This coincides with the proestrus stage. By the time the oocyte has progressed to metaphase II, it is ovulated into the oviduct. This occurs during the estrus stage. If the haploid ovum is fertilized by a sperm, meiosis II proceeds. However, if fertilization does not occur the ovum breaks down. A new cohort of intrafollicular oocytes will begin to mature during the next proestrus phase, which will occur 4 days later.[81]

Primordial follicles are formed in rats during the first three days after birth. Prior to this, oocytes in the ovary are surrounded by somatic cells and arranged in cords. If oocytes do not become integrated into primordial follicles they will undergo apoptosis approximately 3 to 4 days after birth. Shortly after follicle formation, folliculogenesis commences. By day 5-7 after birth, secondary follicles can be observed in the ovaries. By this time, the ovaries have become responsive to gonadotropins. By day 15-17 after birth, antral follicles can be observed. However, rats do not become reproductively mature and capable of ovulation until day 34-35.
after birth. At this point, the pituitary is able to produce the cyclic gonadotropins that will induce ovulation. Any follicles that enter the antral stage before this time will undergo atresia. The cyclic gonadotropins are capable of preventing atresia from occurring in ovarian follicles. The ovarian follicles that are spared the process of atresia will proceed to ovulation. Following ovulation, the granulosa and theca cells form the corpora lutea. If pregnancy does not occur, the corpora lutea will degenerate.\[79\]

In rats, follicular growth occurs over a time span of several weeks. It is estimated that approximately 50 days are required to reach the 8th generation of granulosa cell division, whereas growth from the 8th to the 10th generation occurs in a time span of less than one estrous cycle. By the 4th generation of growth, the granulosa cells become separated from the oocyte by the zona pellucida, formed from glycoproteins that are secreted by the oocyte. The zona pellucide thickens as folliculogenesis proceeds, necessitating that the granulosa cells develop cytoplasmic extensions in order to reach the oocyte. During folliculogenesis, the oocyte grows in size but remains developmentally arrested in the first meiotic prophase. By the 6th or 7th generation of development, a fluid-filled space known as the antrum develops within the layer of granulosa cells. Eventually, the antrum will form a majority of the total volume of large preovulatory follicles. Growth of antral-containing follicles occurs over the span of a few days, in contrast to the several weeks required for a follicle to reach the antral stage. By the time a follicle has reached the antral stage, each follicle is surrounded by an individual vascular network.\[78\]

The period of time required for complete follicular growth can be several weeks. Growth of a primordial follicle to the 8th generation of development can take more than 50 days, during which more than 15 estrous cycles will have been completed. Follicular growth begins slowly, with the granulosa cells of first and second generations follicles displaying doubling rates of
more than 7 days. As the size of the follicle increases, the rate of granulosa cell growth increases. By the time the follicle has progressed to the 8th stage of development (when the antrum develops), granulosa cells display a doubling time of approximately 24 hours. The number of large preovulatory follicles present in the ovary is controlled by feedback mechanisms that are most apparent in the 8th generation of folliculogenesis. At this point, it is estimated that 99% of ovarian follicles will undergo atresia. Atresia will occur if circulating levels of FSH are at baseline levels. However, when FSH levels are elevated in the bloodstream, the follicle will proceed to the 10th generation of development. Growth from the 8th to the 10th generation can occur in less time than is required to complete a single estrous cycle. In the 9th and 10th generation of development, cell proliferation nearest the antrum proceeds rapidly, while cell proliferation in the granulosa cells nearest the basement membrane ceases. Overall, the rate of granulosa cell proliferation rapidly declines. By the 8th or 9th generation of growth, the granulosa and theca cells have functionally matured. By the 10th generation, granulosa cells are no longer capable of proliferating. A follicle is generally completely developed 3 days prior to the ovulation-inducing LH surge. The mature follicle will begin to secrete estradiol. Approximately 24 hours before the LH surge, granulosa cells will display LH receptors. At this point, the follicle is capable of ovulating in response to the LH surge. Ovulation of the follicle will occur approximately 10-12 hours following the LH surge.
Fig. 1.4 Diagram depicting the time course of folliculogenesis in rats. Each black circle represents one generation of follicular development. Follicles will either develop into corpora lutea (CL) or will undergo atresia into secondary interstitial tissue (SI). Granulosa cells become mature during the stage indicated with an asterisk.[78]

**Hormonal Changes Accompanying Folliculogenesis during the Rat Estrous Cycle**

In the first three weeks after birth, female rats display higher FSH levels as compared to males. At this time, plasma LH levels are slightly elevated relative to adult levels. It is hypothesized that estrogen plays a role in follicular growth during this phase.[76]

As proestrus approaches, the theca cells hypertrophy and begin to produce estrogen. The theca cells are considered a major source of estrogen during the rat ovarian cycle. However, both the theca interna and the granulosa cells are necessary for estradiol secretion by the ovary. Ovarian venous plasma levels of estradiol remain at basal levels during estrus and rise during metestrus and diestrus, before reaching peak levels in mid-proestrus.[81] Estradiol levels then decrease until they reach baseline levels during metestrus. Increasing estradiol levels result in a drastic increase in the gonadotropins during proestrus, in what is known as the “preovulatory surge.”[76] Increasing estradiol levels act through a positive feedback mechanism to increase the hypothalamic release of GnRH into the portal vessels that terminate in the anterior pituitary. Estradiol also sensitizes the anterior pituitary to the actions of GnRH, resulting in the secretion of
LH. This LH surge will act on the ovarian follicle to complete maturation and initiate ovulation.[86] The peripheral plasma levels of testosterone and androstenedione follow similar patterns of secretion as estradiol, suggesting that the androgens are also released from the ovary.[81]

Plasma progesterone levels rise dramatically as the gonadotropins (LH, FSH, and prolactin) rise, prior to ovulation. Progesterone levels decline during early estrus before rising again during metestrus.[76] The progestin 20α-hydroxyprogesterone is secreted into ovarian vein blood from the corpora lutea during metestrus. During prooestrus, the corpora lutea again secretes 20α-hydroxyprogesterone, while the granulosa cells of the preovulatory follicle secrete progesterone.[81]

![Fig. 1.5 A graphic depiction of the rat estrous cycle. The shaded blocks indicate the dark periods of a 14 hour light, 10 hour dark photoperiod.][86]
Circulating levels of LH surge during proestrus. This LH surge results in follicular rupture and ovulation. A luteinizing hormone-releasing hormone (LHRH) is present in the rodent hypothalamus that acts to stimulate the secretion of LH from the anterior pituitary. Long portal vessels that arise from the upper infundibulum terminate in the anterior pituitary gland. Therefore LHRH arising in the hypothalamus is released into the portal blood and results in the release of LH into peripheral plasma by the anterior pituitary. Neurogenic stimulation during proestrus triggers the hypothalamus to release LHRH from nerve terminals in the median eminence. This will alter the release of LH from the anterior pituitary, resulting in the LH surge that induces ovulation in the ovary. Other hormones, including oxytocin, neuropeptide Y, and galanin, may also play a role in inducing the anterior pituitary to release LH.

FSH levels simultaneously surge during proestrus. However, unlike LH, a second surge of FSH occurs during early estrus. LHRH will also release FSH from the anterior pituitary although it is unclear if another releasing hormone exists that will affect FSH secretion. Neurohormonal prolactin-releasing factors (PRFs) may exist that will stimulate prolactin release. In addition, large preovulatory follicles are able to exert a negative feedback mechanism on the anterior pituitary, resulting in a decrease in FSH secretion. FSH levels will be able to rise when large preovulatory follicles are not present and therefore unable to suppress FSH secretion. Increased FSH levels allow antral follicles to completely mature into follicles that have the capacity to ovulate. As FSH levels return to normal, the follicles that have passed this “checkpoint” will continue to mature even though FSH levels are no longer elevated. In response to elevated FSH levels, 8th generation follicles secrete inhibin and estradiol that act to decrease FSH secretion by the anterior pituitary. In addition, baseline levels of LH secretion are
required for the maturation of preovulatory follicles. As a result, very few follicles mature in advance of ovulation during each estrous cycle.[87]

The cyclic release of FSH, LH, and prolactin during the estrous cycle is a result of positive and negative feedback from ovarian steroids. Estrogen and progesterone act to decrease LH secretion from late estrus to early proestrus. Estrogen appears to have the most dominant inhibitory effect. The levels of estradiol and progesterone that are secreted from metestrus to early diestrus act on the hypothalamo-pituitary axis to result in basal secretion of LH. Therefore, in ovariectomized rats, LH plasma levels slowly rise in response to the loss of inhibitory control by the ovaries. The LH surge that induces ovulation results from a positive feedback stimulus of ovarian estradiol secreted during diestrus. Therefore the rise in estradiol secretion during diestrus is the stimulus for increased LH secretion in proestrus. The rise in estradiol secretion is simulated by the tonic secretion of LH from metestrus through diestrus. Therefore, tonic LH secretion during diestrus stimulates estradiol secretion by the ovarian follicle, which in turn simulates LH secretion. A surge in LH is limited to the proestrus stage as a result of the increased secretion of progesterone during proestrus. The proestrus surge of progesterone inhibits LH surges following proestrus, but also contributes to the LH surge that occurs during proestrus. Therefore, the progesterone surge that occurs during proestrus initially increases the LH surge induced by estrogen secretion during diestrus, and later inhibits the secretion of LH induced by estrogen. The effects of the second surge of progesterone, that occurs from late metestrus to early diestrus, are unknown. The proestrus surge of LH appears to be the stimulus that increases progesterone secretion during proestrus. The role of prolactin secreted during the estrous cycle is unknown.[81]
The rat brain may also play a role in feedback regulation, because it is able to convert estrone and estradiol into 2- and 4-hydroxylated derivatives known as catechol estrogens. The catechol estrogens may be the active form of estrogen that acts to regulate the secretion of LH and, to a lesser extent, FSH and prolactin.\textsuperscript{[81]}

These hormonal changes can be compared to what occurs in humans, where gonadotropin-releasing hormone (GnRH), released from the hypothalamus, induces the secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary. The secretion of FSH and LH occurs in a cyclic fashion, resulting in a cyclic release of estrogen and progesterone and the cyclic release of oocytes from the ovaries. Estrogen and progesterone act through negative and positive feedback mechanisms to modulate the release of GnRH, FSH, and LH.\textsuperscript{[80]}

*Hormonal Changes in VCD-treated Rats*

Rats and mice treated with VCD display cycling that is disrupted over time. This effect is not attributable to declines in estradiol secretion during proestrus. A rise in FSH levels is also seen in VCD-treated rats and mice, which is a commonly observed following gonadectomy.\textsuperscript{[86]}

*Hormonal Changes Associated with OVX in Rats*

Ovariectomized young (aged 3 month) SD rats display significantly higher levels of LH as compared to ovariectomized middle-aged rats (aged 9-11 months) that were either regularly cycling or had entered persistent estrus prior to ovariectomy. Middle-aged rats that were either cycling or were in persistent estrus prior to ovariectomy displayed similar LH levels. Therefore, following ovariectomy, middle-aged rats display attenuated increases in LH levels. Following
implantation with estradiol capsules, LH levels significantly declined in young SD rats within one day. However, in the middle-aged rats, LH decline was not significant and did not begin until four days after implantation. Therefore the negative feedback response to estradiol is reduced in middle-aged SD rats. A positive feedback response was observed in young rats two days after the estradiol capsule was implanted, wherein LH levels increased. This response did not occur in middle-aged rats, indicating an attenuated positive feedback response of estrogen on LH. Following implantation with testosterone capsules, young and middle-aged rats displayed significant declines in LH levels within one day. However, the decrease was most pronounced in the young rats. Therefore, the negative feedback response to testosterone is also blunted.[83]

**Classifying Rodent Ovarian Follicles**

The Pedersen and Peters (1968) method for classifying oocytes and follicles in the mouse ovary involves classifying the size of the oocyte, the size of the follicle, and the morphology of the follicle. The oocytes are classified into small oocytes (<20 μm in diameter), growing oocytes (20-70 μm in diameter), and large oocytes (final diameter size of 70 μm). Follicles are classified as small, medium, and large. Each size of follicle can be classified according to the number of follicle cells counted in a 5 μm cross-section of the follicle. Therefore, a type 1 small follicle contains an oocyte but no follicle cells. A type 2 small follicle contains a small oocyte and a few follicle cells that are not sufficient to form a complete ring around the oocyte. A type 3a small follicle displays a complete ring of less than 20 follicle cells around the oocyte. A type 3b medium follicle contains 21-60 follicles surrounding a growing oocyte. A type 4 medium follicle contains two layers of 61-100 follicle cells surrounding a growing oocyte. A type 5a medium follicle contains three layers of 101-200 follicle cells surrounding a growing oocyte. A
type 5b large follicle contains 201-400 follicle cells surrounding a large oocyte. A type 6 large follicle contains 401-600 follicle cells surrounding a large oocyte. Interspersed fluid-filled spaces can be observed, that will eventually form the antrum. A type 7 large follicle contains more than 600 follicle cells surrounding a large oocyte, and a large fluid-filled antrum. The oocyte appears to form a hillock. A type 8 large follicle appears similar to a type 7 follicle, except the oocyte has protruded into the antrum and a distinct corona radiata has formed.[85]

The Pedersen and Peters (1968) method appears to only roughly apply to rats, since the number of granulosa cells present in a follicle appears to be related to body size. Therefore follicular diameter appears to be a more reliable method for classifying follicles. This is because a strong positive exponential correlation exists between the number of granulosa cells and follicle diameter. Therefore an increase in follicle diameter by a certain value is correlated with an increase in the area occupied by granulosa cells by the square of that value.[84]

Vaginal Cytology as a Means of Assessing Estrous Cycle Stages

The vaginal epithelium of rats responds to changes in estrogen and progesterone. As a result, the hormonal changes in female rats can be observed by flushing the vaginal cavity with saline and examining the resultant vaginal cytology.[76] Therefore, the estrous cycle stages can be correlated to the types of cells that appear in the vaginal epithelium.[81] For example, in the vaginal epithelium, estradiol acts to increase calcium uptake and activate the calcium-dependent enzyme transglutaminase. Transglutaminase acts to increase the formation of covalent cross-linkages in the vaginal epithelial cells. Therefore the rise in estradiol that begins on diestrus and peaks during mid-proestrus results in the cornification of vaginal epithelial cells.[86]
Vaginal smears can be obtained through lavage. Eyedroppers containing small amounts of saline (0.2-0.25 mL) can be inserted into the first centimeter of the vaginal orifice. The vaginal smears can be placed on microscope slides and observed under light microscopes at magnifications of 100x. During proestrus, round nucleated epithelial cells predominate. These cells appear in clusters during the height of proestrus. A few cornified squamous epithelial cells may also appear during proestrus. In addition, cellular strands may be apparent at this time. During estrus, cornified/keratinized squamous epithelial cells predominate. These cells are unnnucleated, are irregularly shaped, and contain a highly granular cytoplasm. These cells are generally needle-like in appearance. In addition, cells with jagged edges may also be apparent. During metestrus, leukocytes predominate. These cells are small, contain a granular cytoplasm, and display a vesiculated nucleus. Nucleated epithelial cells are also apparent during this stage. Additionally, cornified cells may also appear during metestrus. During diestrus, leukocytes again predominate. This is why metestrus may also be referred to as diestrus 1, while actual diestrus may be referred to as diestrus 2. Diestrus appears similar to metestrus, with the possible addition of small clumps of round nucleated epithelial cells or cellular strands. If vaginal smears are obtained early in the day, the observed epithelial cells may indicate a transition between two stages of the estrous cycle.

For many months before estrous cycle length increases, vaginal cytology may indicate persistently cornified epithelium. Upon entering reproductive senescence and persistent anestrus, vaginal epithelium contains numerous leukocytes.
Reproductive Senescence in Rats

Aged rats begin to display irregular estrous cycles as the number of primordial follicles present in the ovary decreases. Despite this, the number of ova that are ovulated remains constant. Therefore, aged rats are still capable of maintaining normal ovarian function even in the presence of abnormal estrous cycles.\cite{87}

It has been suggested that the number of oocytes released from the ovary occurs at a constant rate until shortly before cyclicity ceases. This is concurrent with a decrease in the numbers of primary and growing follicles. Therefore it is possible that as the numbers of these follicles decrease, the rate of atresia declines. In this case, some follicles that would normally undergo atresia undergo development instead, even if these follicles contain non-viable ova.\cite{76}
In aging rats, the length of the estrous cycle increases as a result of a decrease in progesterone. This presents as an elevated ratio of estrogen to progesterone. As the cycle lengthens, “persistent estrous” can be observed wherein the vaginal epithelium is constantly cornified. When reproductive senescence is reached, the rat is in a state of “persistent anestrus.” Persistent estrus is a result of tonic estradiol levels, resulting from the development of ovarian follicular cysts and a tall columnar endometrial epithelium. The tonic secretion of estrogen cornifies the vaginal epithelium. Low progesterone levels, resulting from a lack of corpora lutea, maintain the state of persistent estrus. During this time, some follicles continue to mature and undergo atresia. Eventually, complete follicular depletion will occur. In the absence of follicles, estradiol levels decrease to the same levels observed in ovariectomized rats. In addition, pituitary tumors increase prolactin levels, resulting in an inhibition of LH secretion.\[82\]

Aging rats display periods of persistent estrus, during which estrogen production remains at tonic levels and progesterone secretion decreases.\[76\] In Sprague Dawley rats the transition to a state of reproductive senescence begins with irregular cycle lengths, which is followed by the cessation of estrous cycles. Finally, the rats display persistent vaginal cornification (PVC).\[77\] The onset of persistent estrus signals the beginning of acyclicity. During persistent estrus, the rat remains in a state of constant sexual receptivity. The tonic production of estrogen cornifies the vaginal epithelium. As the rat ages, the length of the estrous cycle changes and the frequency of ovulation declines.\[76\]

Persistent vaginal cornification is observed as the sustained formation of squamous epithelial. It is a result of continuous estrogen secretion, low progesterone secretion, the lack of LH surges, and the lack of ovulation. This is in contrast to the low estrogen and high LH levels observed in menopausal women. The transition from normal vaginal cytology to persistent
vaginal cornification usually occurs between 6 and 18 months of age. For Sprague-Dawley rats the average onset of this transition occurs at 12 months of age. The tonic secretion of estradiol occurs as a result of numerous follicular cysts. Persistent vaginal cornification can occur after ovulation ceases, even if follicles are continuing to mature and undergo atresia. During this time, injection of progesterone can result in a brief resumption of ovulatory cycles. Mild stress can also transiently reinitiate cyclicity. Eventually the ovaries will become follicle deplete. The increase in LH secretion that is stimulated by estradiol is decreased during persistent vaginal cornification.\textsuperscript{[76]}

In rats, follicle depletion is not the only factor responsible for reproductive senescence. Whereas mice display few oocytes at the beginning of reproductive senescence, rats often display 1,500 to 3,000 oocytes when cycles cease.\textsuperscript{[76]}

When the caloric intake of rats is restricted so that their weight is decreased to half of the weight of control rats, the time until reproductive senescence is extended. Under these circumstances, rats undergo acyclicity and follicular depletion is suspended. This may occur due to increase in the inhibitory effects of estradiol on LH secretion.\textsuperscript{[76]}
TABLE 7. Relation between age of onset of acyclicity and predominant postcyclic vaginal state in different strains of laboratory mouse and rat

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Average age of cycle cessation (months) a</th>
<th>Predominant postcyclic vaginal state</th>
<th>Reference b</th>
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<tr>
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<td>DA</td>
<td>6–7</td>
<td>XX</td>
<td>290</td>
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<td>R × UF1</td>
<td>8–10</td>
<td>XX</td>
<td>967</td>
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<td>8–10</td>
<td>XX</td>
<td>661</td>
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<td>XX</td>
<td>216</td>
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<td>XX</td>
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<td>XX</td>
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<td>XX</td>
<td>949</td>
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</table>

From ref. 305, with permission.

*Ranked according to age of cessation of cyclicity.

†Age when 50% of the animals have ceased cycling.

‡PVC, persistent vaginal cornification; RP, repeated pseudopregnancy; PA, persistent anestrus.

§Pers. comm., personal communication; unpubl., unpublished data.

X indicates that this state occurs but less frequently than XX.

Fig. 1.7 Cyclicity data of various laboratory mouse and rat strains.[76]

**Follicular and Hormonal Changes during the Menstrual Cycle in Humans**

During their reproductive lifetime, humans display menstrual cycles that are typically 28 days in length.[76] Humans are also born with a finite pool of primordial follicles that may remain in the arrested prophase of meiosis for up to 50 years before the onset of menopause.[78] As menopause approaches, menstrual cycle length becomes irregular. At this time, cycle length may become longer as ovulation fails to occur, or shorter as the follicular phase becomes shorter. During the period of irregular menstrual cycles, follicle loss accelerates. This process culminates in menopause, when the finite pool of primordial follicles that was established before birth has been exhausted.[76]
The follicular phase lasts 14-15 days from the first day of the onset of menses. During this time a cohort of primordial follicles begins to grow. Most of these follicles will become atretic, while only one of these follicles will continue to grow. The follicular phase ends with ovulation, when the follicular wall ruptures and the ovum is released into the peritoneal cavity. Thus the luteal phase begins. This period lasts for approximately 14 days until the beginning of the next menses. During the luteal phase, the granulosa cells increase in size to form the corpus luteum.[109]

The onset of the follicular phase begins in response to a surge in FSH. FSH will stimulate growth of granulosa cells and transformation of stroma into thecal cells. The growing follicle will begin to secrete estrogens. Estrogen sensitizes the follicular tissue to the effects of FSH and also suppresses further FSH secretion; therefore, the first follicle to secrete estrogen may display a greater response to FSH and undergo development while suppressing the FSH that would allow other follicles in the cohort to grow. As the other follicles undergo atresia, they may secrete ovarian androgens. Increasing estrogen will continue to stimulate growth of this single follicle, as well as release of LH. At the time of ovulation, increasing estrogen levels stimulate a surge in LH. A concurrent rise in FSH, a decline in estrogen secretion, and the onset of progesterone secretion precede ovulation. LH and FSH secretion from the anterior pituitary is directly stimulated by hypothalamic releasing hormones (LHRH and FSHRH). Throughout the menstrual cycle, LH and FSH are released in a pulsatile manner.[109]

In the luteal phase, the corpus luteum secretes progesterone and 17-α-hydroxyprogesterone. This is followed by a secondary rise in estrogen. Simultaneously, FSH and LH secretion is suppressed. The activity of the corpus luteum continues for 8-9 days
following ovulation. At the end of the luteal phase, estrogen and progesterone levels decline, resulting in the onset of menstruation.⁹⁰

Fig. 1.8 Ovarian and hormonal changes that occur during a normal human menstrual cycle.⁹⁰

**Causes of Menopause in Humans**

Before birth, human females have developed all of the primordial follicles that will ever be stored in the ovaries. By the onset of menopause, almost all ovarian oocytes have been depleted, resulting in reproductive senescence and the cessation of menstrual or fertility cycles. This follicle depletion occurs at an exponential rate until the age of 35, when the rate at which follicles are lost increases dramatically. By the onset of menopause, the pool of ovarian follicles has been completely depleted. Studies reveal that cycling women display follicular reserves of 1,000 to 2,500 follicles, while perimenopausal women display a few hundred follicles, and
menopausal women display none. During the menopausal transition, an irregular pattern of menstrual cycles is observed. This may be accounted for neuronal changes, including a decrease in the numbers of hypothalamic neurons with increasing age and post-menopausal hypertrophy of neurons. It has also been hypothesized that a minimum number of ovarian follicles are necessary to stimulate the estrogen secretion that triggers the gonadotropin release preceding ovulation. Therefore as follicle numbers decline toward this “threshold” value, the number of estrogen-producing/growing follicles might fluctuate, resulting in cycles of varying length.[76]

Fig. 1.9 Graph demonstrating the biexponential relationship between total number of follicles and age in humans.[76]

Endocrinology of Menopause in Humans
In post-menopausal women, estradiol and progesterone production by the ovary ceases. In the ten years preceding menopause, there is an increase in FSH levels and a decrease in estradiol secretion during the follicular phase. The rise in FSH is most likely a result of the decline in estrogen secretion and a decline in the plasma levels of the FSH-inhibiting inhibin. During this ten-year period, fertility cycles become prolonged and are generally anovulatory. Plasma LH levels also increase relative to the levels observed in young adult women. Progesterone secretion, like estrogen secretion, declines to low levels during this period. During the menopause transition, follicular phases are usually shortened, which may be a result of increased FSH secretions. This would accelerate the maturation of any remaining ovarian follicles. Post-menopause, the pituitary gland continues to respond to GnRH, even though the concentration of GnRH declines.\[^{76}\]

Progesterone acts synergistically with estradiol to affect cycle length. In middle-aged rats the progesterone secretion that occurs midway through the estrous cycle decreases by 30-50\%, regardless of whether the estrous cycle is prolonged or normal. However in young adult rats plasma progesterone levels remain elevated with longer estrous cycles (5-day) as compared to shorter estrous cycles (4-day). Progesterone implants in middle-aged rats can act to delay the onset of irregular cyclicity and acyclicity. Therefore the decline in progesterone that occurs with aging may result in prolonged estrous cycles prior to acylcicity. Elevated estradiol levels are linked to prolonged estrous cycles between 10 and 12 months of age in rats. In addition, the period of time during which vaginal cells are cornified prior to ovulation increases. Therefore, the ratio of estrogen to progesterone may be elevated prior to ovulation in middle-aged rats. Prior to ovulation, testosterone and androstenedione are also elevated in middle-aged rats.\[^{76}\]
Before acyclicity commences, middle-aged rats display reduced LH levels during the peak of proestrus. In addition, the LH surge that occurs prior to ovulation is delayed by 2 to 3 hours. *In vitro* studies demonstrate that application of GnRH to the rat pituitary results in an LH secretion that is smaller in young adult rats as compared to middle-aged that are still cycling.[76]

The surge in prolactin that occurs before ovulation is decreased in cycling middle-aged rats relative to young adult rats. It is unclear whether FSH secretion changes with age.[76]

With reproductive senescence, plasma estradiol levels decrease to those levels observed in ovariecтомized rats. This occurs because there are no longer any developing follicles present in the ovaries. In addition, progesterone levels are decreased as a result of the lack of copora lutea. Plasma LH levels are not increased during persistent anestrus in rats because pituitary mammotropic tumors result in high levels of LH-inhibiting prolactin. These tumors begin to develop with age, and secrete prolactin at the onset of persistently cornified vaginal epithelium.[76]

**Comparison of Natural Menopause to Ovariectomy in Humans**

The endocrine changes observed during natural menopause also occur post-ovariectomy. However, the post-menopausal ovary continues to secrete low levels of testosterone from the ovarian interstitial cells. Since changes in the adrenal secretion of DHEA and its sulfated conjugate are independent of changes in the ovary, changes in the levels of these steroids may not be analogous between women who have undergone natural or artificially-induced menopause.[76]

If only one ovary is removed, the remaining ovary will compensate by undergoing hypertrophy. This hypertrophy will impact fertility and result in smaller litters, a decrease in the
number of implanted embryos, and a decrease in the number of offspring. The hypertrophied ovary will increase the number of oocytes at ovulation by initially releasing the same number of ova that would normally be released by both ovaries. However, the rate of loss of oocytes from the hypertrophied ovary appears to be the same as if both ovaries remain intact. During the period of estrus in young adult rats that have undergone unilateral ovariectomy, FSH levels will increase relative to control rats. This also occurs during the metestrus period for middle-aged rats that have undergone a unilateral ovariectomy. This rise in FSH is most likely due to a decrease in inhibin secretion, resulting from follicle depletion. A sustained, elevated secretion of FSH may result in an increase in the number of ovulations by the hypertrophied ovary.^[76^]

Post-ovariectomy, the elevation in LH declines with age. Young rats (4-5 months) that are ovariectomized display higher elevations of LH than middle-aged (12-15 months) that are ovariectomized. LH surges can be reduced in ovariectomized rats by the administration of estradiol. Young rats display greater depressions in LH, following estradiol administration, as compared to middle-aged rats. This would indicate that without the presence of ovarian steroids, increasing age results in a decline in the ability to secrete LH. Acyclic rats that are ovariectomized display greater reductions in the amplitude and frequency of LH surges, as compared to irregularly cycling middle-aged rats that are ovariectomized. Irregularly cycling middle-aged rats that are ovariectomized display greater reductions in LH pulses as compared to rats that are acyclic prior to ovariectomy. This data also serves to indicate that prior to the cessation of cyclicity, the regulation of gonadotropin secretion has already begun to change. The decline in the pre-ovulatory LH surge occurs prior to any changes in the estrous cycle. The onset of this decline can be used to predict the onset of acyclicity.^[76^]
Table 6. Steroids and gonadotropins in the human female (representative values)

<table>
<thead>
<tr>
<th>Plasma levels</th>
<th>Young adult</th>
<th>Follicular phase</th>
<th>Luteal phase</th>
<th>Postmenopause</th>
<th>Ovariectomy at middle age</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estrone (E₁), pg/ml</td>
<td>60–200a</td>
<td>60–100b</td>
<td></td>
<td>30–60b</td>
<td>50b</td>
</tr>
<tr>
<td>Estradiol (E₂), pg/ml</td>
<td>50–350a,b</td>
<td>100–200</td>
<td></td>
<td>10–20c,d,e</td>
<td>10–20d,e</td>
</tr>
<tr>
<td>Estriol (E₃), pg/ml</td>
<td>10–30</td>
<td>3–10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Progesterone (P₄), pg/ml</td>
<td>300–2,000a</td>
<td>2,000–20,000b</td>
<td></td>
<td>150–200b</td>
<td>180b</td>
</tr>
<tr>
<td>Testosterone (T), pg/ml</td>
<td>300–400a,d</td>
<td>250</td>
<td>250</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortisol, ng/ml</td>
<td>150</td>
<td>3–5**</td>
<td>0.5–2.2**</td>
<td>No effect</td>
<td></td>
</tr>
<tr>
<td>Dehydroepiandrosterone, µg/ml</td>
<td>0.1–1.0b</td>
<td>0.1f</td>
<td>0.2–0.4f</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Luteinizing hormone (LH), µg/ml</td>
<td>0.2–0.5a</td>
<td>0.1–0.2a</td>
<td>1.0–1.5a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Follicle-stimulating hormone (FSH), µg/ml</td>
<td>1–20m</td>
<td>6–8f,g</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* From ref. 407.  
  † From ref. 76.  
  ‡ From ref. 976.  
  † From ref. 393.  
  No marked variations across the menstrual cycle.  
  † From ref. 393.

Fig. 1.10 Table depicting the basal levels of steroids and gonadotropins observed in human females across the lifespan.[76]

Postmenopausal Bone Changes in Humans

The loss of estrogen in postmenopausal women is associated with detrimental changes in bone. For example, postmenopausal women who experience hip fractures often display lower free plasma estradiol and progesterone levels than postmenopausal women who do not develop hip fractures. In addition, estrogen replacement in postmenopausal women results in a dose-dependent decrease in fracture risk. However, not all bone changes can be accounted for by estrogen depletion, suggesting that bone loss is multifactorial.[76]
VINYLCYCLOHEXENE DIEPOXIDE (VCD)

**Industrial Use of VCD and the related compound VCH**

Vinylcyclohexene diepoxide (VCD) is also known as 4-vinyl-1-cyclohexene diepoxide, 4-vinylcyclohexene diepoxide, 4-vinyl-1,2-cyclohexene diepoxide, 1-vinyl-3-cyclohexene dioxide, 4-vinylcyclohexene dioxide, 1,2-epoxy-4-(spoxyethyl)cyclohexane, 1-epoxyethyl-3,4-epoxycyclohexane, vinylcyclohexene dioxide, 3-epoxyethyl-7-oxabicyclo[4.1.0]heptane, 3-oxiranyl-7-oxabicyclo[4.1.0]heptane, Chissonox 206, EP-206, ERLA-2270, ERLA-2271, NCI-C60135, and Unox Epoxide 206.[13, 15]

VCD (C₈H₁₂O₂) has a molecular weight of 140.18 g/mol, a density of 1.094 g/mL at 25°C, a refractive index of n₂₀/D 1.479 (1.477 literature), and a flash point of 107°C.[13] VCD is a light brown-yellow liquid that is stable unless exposed to strong oxidizing agents.[22] VCD is soluble in water, but is slowly hydrolyzed in water. It is reactive with active hydrogen compounds such as alcohols and amines.[15] It is also considered a slightly water polluting substance (WGK 1).[13]

![Structure of 4-vinyl-1-cyclohexene diepoxide](image)

VCD is used as a reactive diluent for other diepoxides as well as for epoxy resins.[16] VCD is also used to make an embedding mixture for electron microscopy, in a process that involves mixing 10 grams of VCD with other chemicals. This embedding mixture is recommended for use in preserving the vacuolar system in plant cells and for reducing
plasmolysis.[65] VCD is used as a chemical intermediate for condensation with dicarboxylic acids, as a reactive diluent for other diepoxides, and as a reactive diluent for bisphenol-A- and epichlorohydrin-derived epoxy resins. It has been proposed for use as a monomer like in the preparation of polyglycols that contain unreacted epoxy groups.[15]

VCD is formed as a result of the epoxidation of VCH with peroxyacetic acid. VCH is produced from the catalytic dimerization of 1,3-butadiene. VCH is used as a chemical intermediate for producing flame retardants, flavours, fragrances, and polyolefins. VCH has also been used as a solvent.[16] VCH is ovotoxic in mice, following in vivo bioactivation to its epoxide metabolites VCH-1,2-epoxide, VCH-7,8 epoxide, and VCD.[38] The ultimate ovotoxic effects of VCH in vivo are thought to result from the actions of VCD.[39]

It is unknown how many individuals are occupationally exposed to VCD.[16] The National Institute for Occupational Safety and Health estimates that between 1981 and 1983, approximately 1,997 workers in the U.S. were potentially exposed to VCD.[19] However, no major companies, like 3M or General Electric, who are committed to the High Production Volume (HPV) challenge as of January 31, 2008 use VCD. This indicates that VCD is not used or imported in high volumes of greater than 1 million pounds or more per year.[64] In addition, VCD is not listed in the Environmental Protection Agency’s Chemical Information Rules in 40 CFR 712 as of July 1, 2005; however, 4-vinylcyclohexene (VCH) was listed as of January 11, 1990. This document defines the procedures chemical manufacturers and processors must complete in order to report their production, use, and exposure rates on any chemical listed by the Interagency Testing Committee as being recommended for testing.[66]

**Metabolism and Toxicity of VCD in Humans**
VCD is a skin, eye, and respiratory system irritant.\cite{19} It is considered toxic by inhalation, skin contact, and ingestion. There is also the possibility that the side effects of VCD may be irreversible, if it is consumed by any of these routes. Even though there is only a slight risk of inhaling VCD, it is suggested that users do not breathe in VCD fumes.\cite{13, 15} Individuals exposed to VCD are also encouraged to avoid contact with VCD through the skin and eyes.\cite{13} VCD is mildly to moderately irritating to skin; however, VCD was found to be a severe skin irritant in one case where a human wore shoes contaminated with VCD.\cite{15}

Workers exposed to VCD and other compounds at an industrial facility complained of eye, nasal, skin, and respiratory irritation. These workers exhibited antibodies to aliphatic diisocyanate, VCD, trimellitic anhydride, and a component of \textit{n}-octyl-\textit{n}-decyl-trimellitate. Those workers with the highest exposure exhibited the worst symptoms. Since VCD has a low vapor pressure of less than 0.1 minutes at 20 °C, it is assumed that atmospheric concentrations of VCD would be insignificant. Based on experimental animal carcinogenicity tests, it is assumed that a dose for humans of 2.7 \( \mu \)g/kg/d VCD would be considered a 1x10^{-5} cancer risk under California Proposition 65 guidelines.\cite{24}

Disposable latex or PVC gloves are permeable to VCD. In one instance, VCD at a concentration of 0.25% in acetone, caused a 3+ blistering reaction on human skin. This blistering action occurred even when VCD was allowed to dry on a disposable latex glove. Twenty other humans failed to exhibit such a reaction, indicating that allergic sensitization and dermatitis in reaction to VCD only occurs in some humans.\cite{14}

There is no direct evidence that VCD is carcinogenic in humans; however, VCD is considered a possible human carcinogen due to demonstration of carcinogenicity in animals.\cite{15} VCD targets the thymus, blood, and the male reproductive system of humans.\cite{22} VCD also
alters T cell proliferation and decreases leukocyte counts. This occurs because alkylating compounds such as VCD are selectively active towards rapidly dividing cells.

Because VCH is ovotoxic in mice following *in vivo* conversion to its monoepoxide and diepoxide forms, it is also important to establish whether VCH metabolism occurs in humans. In human liver microsomes, VCH is only epoxidized to VCH-1,2-epoxide (the major product) and VCH-7,8-epoxide when an epoxide hydrolase inhibitor is administered. This implies that there is epoxide hydrolase activity on VCH epoxides in the human liver microsome. There is no difference between male and female human hepatic microsomes in their epoxidation of VCH. The rate of VCH epoxidation in human microsomes was less than the rate in mice, and on par with the rate in rats. This suggests that humans are less susceptible than mice to ovotoxicity induced by VCH. Incubations of VCH with human CYP2E1 enzyme results in the formation of VCH-1,2-epoxide and VCH-7,8-epoxide but not VCD. Human CYP2A6 forms very little VCH epoxides and no VCD. Human CYP2B6 forms more VCH epoxides than CYP2A6, with more VCH-7,8-epoxide forming than VCH-1,2-epoxide. No VCH epoxides are made by human CYP3A4.

**Mutagenicity of VCD**

VCD is mutagenic in the TA98 (tester for frameshift mutations) and TA100 (tester for base-pair substitution mutagens) strains of *Salmonella typhimurium* in both rat-liver enzyme-activated and non-activated bacterial plate assays. This VCD mutagenic assay was conducted with 0.05 and 10.00 mg of VCD.

VCD is mutagenic to the non-activated base-pair indicator strains TA100 and TA1535 of *Salmonella typhimurium*, at doses of 15-60 μmoles per plate. Compared to cyclopentane oxide,
4,5-epoxycyclohexene, cyclohexane oxide, norbornane oxide, 3,4-epoxycyclohexene, and oxaspirooctane, VCD is the most mutagenic in TA100. However, in TA1535 VCD is only more mutagenic than 3,4-epoxycyclohexene. At a dose of 48 μmoles per plate VCD inhibits 6% of TA100 and 8% of TA1535. At a dose of 60 μmoles per plate VCD inhibits 12% of TA100 and 15% of TA1535.[73]

In the *Salmonella typhimurium* strain TA100, 100 μL of VCD was found to create 632±67 mutants per plate, the highest among electron microscopy embedding medias including ipon, araldite, DER-736, HXSA, NSA, DMP-30, and DMAE. VCD was also found to be mutagenic to TA98. The mutagenicity of VCD was greatly increased by exposure to mixed oxidase enzymes. The mutational activity per n mole of VCD was found to range between 0.004 and 0.015 revertants. This is comparable to the related mutagen of 1,2-epoxy butane, which is a known carcinogen. Since a compound is not labeled a known carcinogen until epidemiological studies verify that exposed individuals exhibit elevated cancer rates, VCD is labeled a suspected carcinogen.[74]

Another study found VCD to be mutagenic in *Salmonella typhimurium* strains TA98, TA100, and TA1535, both with and without activation. However, VCD was only mutagenic in strain TA1537 following activation. In addition, VCD will induce resistance to trifluorothymidine in mouse L5178Y/TK cells. Furthermore, VCD will induce sister chromatid exchanges and chromosomal aberrations in Chinese hamster ovarian cells.[19]

**Metabolism and Toxicity in Rodents-VCH**

Because VCH can be metabolized to VCD *in vivo*, knowledge of the toxic effects of VCH may be relevant to understanding the *in vivo* effects of VCD.[39] In 14 day, prechronic
toxicity studies, male and female Fischer 344 rats (7 weeks old) and male and female B6C3F₁ mice (8 weeks old) were administered 300, 600, 1250, 2500, or 5000 mg/kg VCH in corn oil or vehicle control by gavage. Fischer 344 rats that were administered 1250, 2500, or 5000 mg/kg VCH by gavage died before 14 days. Before death these animals were lethargic, had messy bottoms, exhibited tremors, had soft stools, and were wobbly. B6C3F₁ mice that were administered 2500 or 5000 mg/kg VCH and 3 out of 5 males that received 1250 mg/kg VCH by gavage died before 14 days. These animals exhibited tremors and were lethargic before they died.¹⁷

In 13 week subchronic toxicity studies, male and female Fischer 344 rats (7 weeks old) and male and female B6C3F₁ mice (8 weeks old) were administered 50, 100, 200, 400, or 800 mg/kg VCH in corn oil or vehicle control by gavage. For rats that received VCH by gavage for 13 weeks, mean body weights were lower in males and females receiving 800 mg/kg VCH. One male rat administered 400 mg/kg VCH and 1 female rat administered 800 mg/kg VCH died before the end of the 13 weeks. Male rats dosed with 50, 100, 200, 400, or 800 mg/kg VCH by gavage for 13 weeks exhibited hyaline droplet degeneration of the kidney proximal convoluted tubules. One male and 3 female rats receiving 800 mg/kg VCH by gavage for 13 weeks exhibited inflammation in the submucosa of the nonglandular portion of the stomach. Nine out of 10 male mice and 5 out of 10 female mice administered 1200 mg/kg VCH by gavage for 13 weeks died. Two out of 10 female mice administered 300 mg/kg VCH by gavage for 13 weeks died. Due to the high death rates of rats and mice, these studies were found to be inadequate indicators of carcinogenicity. In all 10 female mice administered 800 mg/kg VCH by gavage for 13 weeks, the number of primary and mature graafian follicles was reduced. It is unknown whether females in the lower dosage groups exhibited such a follicle loss as well.¹⁷
In 103 week oral toxicity studies, female B6C3F₁ mice (8 weeks old) were administered 200 or 400 mg/kg VCH in corn oil or vehicle control by gavage. Female B6C3F₁ mice administered 400 mg/kg VCH by gavage for 103 weeks had reduced survival probabilities after 32 weeks. Female mice had slightly lower body weights than controls, after 20 weeks. Female mice also developed ovarian neoplasms including mixed benign tumors, granulosa-cell tumors, and granulosa-cell carcinomas (which metastasized to the lungs). Female mice also tended to have increased development of bilateral tubular-cell hyperplasia and granulosa cell hyperplasia in the ovary. Female mice also exhibited tubular adenomas, luteomas, and papillary adenomas in the ovaries. Outside of the ovary, female mice exhibited increased occurrences of mild acute inflammatory lesions, epithelial hyperplasia of the forestomach, congestion of the lungs, congestion of adrenal glands (at 400 mg/kg VCH only), capsular adenomas of the adrenal gland, and cytologic alteration of the adrenal cortex.¹⁸

In 13 week inhalation toxicity studies, male and female Sprague Dawley rats (22 days old) and B6C3F₁ mice (36 days old) were exposed to VCH 65 times by inhalation over a 13 week period at concentrations of around 53, 250, 1000, and 1500 ppm. Mice exposed to VCH exhibited significant compound-related mortality. All of the male mice and 5 out of 10 female mice exposed to 1000 ppm VCH died on day 11 or 12 of treatment. Rats exposed to VCH at levels of 250 and 1000 ppm became lethargic and slow to respond to stimulus. They occasionally had clear or bloody discharge from their mouth and nose and stained fur. Male and female rats exposed to 1500 ppm VCH lost weight compared to controls. Male and female rats exposed to 1000 or 1500 ppm VCH exhibited increased liver weights, while only the males in these groups had increased kidney weights. All male rats displayed increased hyaline droplet accumulation in the renal proximal tubules, that may not be compound related. Female mice
exposed to 1000 ppm VCH demonstrated loss of all types of ovarian follicles, while a few rats exposed to 1500 ppm VCH exhibited a loss of corpora lutea that was deemed a spurious event. A few male mice displayed lymphoid atrophy, which is a common nonspecific stress response.[25]

VCH mainly concentrates in adipose tissue in rodents. The ethylene carbons of VCH are eliminated in urine and through respiration. When metabolized, VCH is oxidized to its monoepoxide products.[16]

**Metabolism and Toxicity in Rodents-VCD**

The oral LD$_{50}$ value is 2.13 g/kg for rats and the inhaled LC$_{50}$ value is 800 ppm/4 hours.[19]

In dermal studies, male and female Fischer rats administered 0.1 mL of 500 mg/mL VCD in acetone, and male and female B6C3F$_1$ mice administered 0.01 mL of 500 mg/mL VCD in acetone, absorb approximately 30% of the dermally-applied VCD over 24 hours. In addition, by 24 hours 70-80% of the absorbed dose is eliminated from the body, primarily through urine. Of the VCD that remains in the body, no tissue contains more than 1% of the applied dose. However, the liver, muscle and adipose tissue of the rat contains 0.5-1.6% of the absorbed dose, while the liver, muscle, and adipose tissue of the mouse contains 1.2-2.9% of the absorbed dose.[19] Therefore VCD can be absorbed through the skin of rodents, and is eliminated primarily through the urine.[16]

In single-dose dermal studies, male and female F344/N rats (6 weeks old) were administered VCD in acetone or vehicle control at concentrations of 198, 388, 773, or
1,568 mg/kg. In addition, a single dose of VCD at a concentration of 3,074 mg/kg was administered neat. All rats survived following the single administration of dermal VCD. However, rats administered 773, 1,568, and 3,074 mg/kg VCD displayed compound-related decreases in activity.[19]

In single-dose dermal studies, male and female B6C3F1 mice (8 weeks old) were administered VCD in acetone or vehicle control at concentrations of 338.3, 671.6, 1,378, or 2,741 mg/kg. In addition, a single dose of VCD at a concentration of 5,487 mg/kg was administered neat. All mice treated with 5,487 mg/kg VCD, one of the female mice treated with 2,741 mg/kg VCD, and one of the female mice treated with 671.6 mg/kg VCD died. VCD administration resulted in decreased activity, rapid respiration, and skin irritation at the site of VCD application[19]

In 14-day dermal studies, male and female F344/N rats (7 weeks old) and male and female B6C3F1 mice (9 weeks old) were administered VCD in acetone. Male rats were administered VCD at concentrations of 0, 35, 68, 139, or 289 mg/rat in acetone or 358 mg/rat neat. Female rats were administered VCD at concentrations of 0, 27, 57, 112, 211, or 290 mg/rat. Male mice were administered VCD at concentrations of 0, 3, 5, 10, 21, or 43 mg/mouse. Female mice were administered VCD at concentrations of 0, 2, 5, 10, 19, or 37 mg/mouse. Rats administered VCD at concentrations of 112 mg/kg or higher died. Male rats administered 139 mg/rat VCD and females given 112 mg/rat developed congestion and/or hypoplasia of the bone marrow and acute nephrosis. Rats receiving 139 and 112 mg/rat VCD developed epidermal necrosis and ulceration, epidermal hyperplasia, and hyperkeratosis. All mice administered VCD at a concentration of 37 or 43 mg/mouse, 3 out of 5 male mice administered 21 or 19 mg/mouse, and all female mice administered 21 or 19 mg/mouse died
before the end of the studies. B6C3F₁ mice administered 10 and 21 mg/mouse in males and 19 mg/mouse in females developed epidermal and sebaceous gland hyperplasia, hyperkeratosis, and ulceration.\[19\]

In 13 week dermal studies where VCD was applied at concentrations of 3.75, 7.5, 15, 30, or 60 mg/rat in acetone, male and female Fischer (F344/N) rats (10 weeks old) exhibited lower body weights than controls, hyperplasia of sebaceous glands, acanthosis, parakeratosis, and hyperkeratosis of the stratified squamous epithelium. In 13 week dermal studies where VCD was applied at concentrations of 0.625, 1.25, 2.5, 5, or 10 mg/mice VCD in acetone, male and female B6C3F₁ mice (9 weeks old) exhibited increased kidney and liver weights, acanthosis, and hyperkeratosis. All of the 10 mg/mice VCD dosed female mice and 4 out of 10 female mice in the 5 mg/mice VCD dosed group exhibited ovarian atrophy.\[19\]

In another set of 13 week dermal studies male and female Fischer rats and male and female B6C3F₁ mice were administered VCD in acetone at concentrations of 0, 3.75, 7.5, 15, 30, or 60 mg/mL VCD. Rats received three consecutive applications of 0.1 mL of VCD, while mice received a single application of 0.1 mL of VCD. Male rats displayed 14% lower mean body weights following treatment as compared to controls, while female rats displayed 9% lower final average body weights as compared to controls. All rats displayed diffuse hyperplasia of sebaceous glands, parakeratosis, and hyperkeratosis of the stratified squamous epithelium at the site of VCD application. Male and female mice displayed compound-related increases in kidney and liver weights. Male mice administered 60mg/mL VCD displayed acanthosis of the stratified squamous epithelium at the site of VCD application. Four out of 10 female mice in the administered 5mg of VCD displayed a decrease in the number of ovarian follicles. This study suggests that VCD is a direct irritant at the site of skin exposure.\[20\]
In 13 week gavage studies, where VCD was administered at concentrations of 62.5, 125, 250, 500, or 1000 mg/kg in corn oil, Fischer rats (7 weeks old) receiving 500 and 1000 mg/kg exhibited lower body weights than controls. Rats receiving 250, 500, or 1000 mg/kg VCD also exhibited increased salivation. Rats receiving 1000 mg/kg VCD had larger liver and kidney weights, lower lymphocyte counts, and degeneration and necrosis of tubular cell epithelium in the kidney cortex. All rats receiving VCD (except those in the 62.5 mg/kg group) developed hyperplasia and hyperkeratosis of the forestomach stratified squamous epithelium. At 500 and 1000 mg/kg VCD rats exhibited tubular cell regeneration in the renal cortex. In mice undergoing the same gavage experiment, all mice receiving VCD has lower body weights than controls and increased kidney weights. The B6C3F1 mice (6 weeks old) administered 1000 mg/kg VCD had increased liver weights. Except for the group administered 62.5 mg/kg VCD, all mice exhibited hyperplasia and/or hyperkeratosis of forestomach stratified squamous epithelium. Male mice exhibited a decrease in testis size (500 and 1000 mg/kg VCD) and a decrease in the number of germinal epithelial cells in the seminiferous tubules (250, 500, and 1000 mg/kg VCD). Female mice exhibited ovarian atrophy and uterine atrophy (1000 mg/kg VCD only).[20]

In 15 month and two year dermal studies, male and female Fischer rats (7-8 weeks old) were administered VCD in acetone at concentrations of 0, 15, or 30 mg/rat, while male and female B6C3F1 mice (8-9 weeks old) were administered VCD in acetone at concentrations of 0, 2.5, 5, or 10 mg/mouse. After 15 months of dermal application of VCD at concentrations of 15 or 30 mg/animal in F344/N rats, body weights were 3-10% lower than control rats. At a VCD concentration of 30 mg/animal, two out of ten male rats developed squamous cell carcinoma. Male and female rats dosed with 30 mg/animal VCD developed hyperkeratosis and acanthosis. Male and female rats dosed with 15 mg/animal VCD developed acanthosis. Body weights and
survival probabilities were reduced for rats after 2 years of dermal VCD application. These animals also developed basal cell adenomas, basal cell carcinomas, squamous cell papillomas, squamous cell carcinomas, sebaceous gland adenomas, acanthosis, and sebaceous gland hypertrophy. In a few rats, these carcinomas metastasized to the lung or other organs. After 15 months of dermal VCD application (2.5, 5, or 10 mg/animal) in B6C3F1 mice, body weights were 3-13% lower than control rats. These mice developed sebaceous gland hyperplasia, sebaceous gland hypertrophy, acanthosis, and hyperkeratosis. Mice in the 5 or 10 mg VCD groups developed squamous cell papillomas and carcinomas. All of the female mice developed ovarian tissue atrophy and most developed tubular hyperplasia of the ovarian surface epithelium. In the highest dosed female mice, ovarian granulosa tumors were seen. After 2 years of dermal VCD application, mice had lower body weights than controls and lower survival probabilities. These mice also exhibited increased incidences of squamous cell carcinomas, acanthosis, hyperkeratosis, and necrotizing dermal inflammation. Female mice in the 2 year studies also developed atrophy, tubular hyperplasia, and granulosa cell tumors of the ovaries. For female mice dosed with 5 mg/animal VCD, there was an increase in the incidence of alveolar/bronchiolar adenomas or carcinomas. The overall conclusions of this dermal toxicity study are that VCD is carcinogenic to skin in male and female rats and mice.\textsuperscript{[19, 21]}

All of the toxicity findings support the conclusion that there is clear evidence of carcinogenic activity of VCD in male and female F344/N rats as evidenced by squamous and basal cell neoplasms in dermal applications. There is also clear evidence of carcinogenic activity of VCD in male and female B6C3F1 mice as evidenced by squamous cell carcinomas and ovarian neoplasms.\textsuperscript{[19]}
The ovotoxic index (OI) is defined as the lowest concentration of an ovotoxic chemical that will result in a 50% loss of primordial follicles following 15 daily doses. A lower OI likely indicates a more ovotoxic chemical. For VCD the OI is 8.6 when administered to rats at a concentration of 80 mg/kg. The OI is 6.8 in mice. VCD is therefore much less ovotoxic than 9,10-dimethylbenzanthracene (DMBA), 3-methylcholanthrene (3-MC), and beno[α]pyrene (BaP).[67]

VCD can be absorbed through the skin of rodents, and causes benign and malignant skin neoplasms. VCD is eliminated through urine. In female rodents, concentrations of VCD are higher in the ovary. When metabolized, VCD is hydrated to a mixture of glycols and glutathione conjugates.[16]

Carcinogenicity may be monitored through the identification of DNA adducts formed in response to VCD administration. The reaction of VCD towards DNA is tested through 32P-postlabeling assay of VCD-DNA adducts. Adducts were evident in female ICR mouse (21-24 grams) skin that was painted with VCD at concentrations of 14.4, 17, 36, 51, 90, 153, and 225 μM. The adducts appeared in a dose-dependent manner that showed biphasic kinetics. Adducts were not evident at a VCD concentration of 17 μM, which is most likely due to the variability in the response of individual mice to VCD. In addition, adducts formed at 14.4 and 36 μM appeared at similar relative adduct labeling (RAL) values, which indicate the extent of DNA modification. Overall, this work indicates that exposure of mice to VCD results in the formation of apparently pre-mutagenic DNA adducts.[68]

Studies evaluating the toxicity of VCD administered through an intraperitoneal (ip) or intramuscular (im) route have not been reported in the literature.
Detoxification of VCH and VCD by Cytochrome P450 Enzymes

Cytochrome P450 isoenzymes (CYPs) are enzymes that catalyze the metabolism of many compounds, including drugs and xenobiotics. They are called isoenzymes because each enzyme is encoded by a different gene. These enzymes are mostly found in the liver, where they catalyze reactions such as N-dealkylation, O-dealkylation, S-oxidation, epoxidation, and hydroxylation. CYPs consume one molecule of oxygen to produce an oxidized substrate, so they are often called mixed function oxidases or mono-oxidases. The different isoenzymes are categorized by family, subfamily, and individual gene. For example, CYP2A6 belongs to the CYP2 family, the CYP2A subfamily, and is derived from gene 6.[1]

When a drug is repeatedly administered, the CYP450 isoenzymes can become induced. The drug will usually cause an increase in the rate of production of the isoenzyme. It is hypothesized that the drug binds to the ligand binding domain (aromatic hydrocarbon (Ah) receptor) of a soluble protein. This causes a conformational change in the Ah receptor, which will transport the drug-Ah complex to the nucleus via an Ah-receptor nuclear translocator. In the nucleus, Ah-receptor response elements will bind the drug-Ah complex and promote transcription of the corresponding gene. Drugs can also inhibit the CYP450 isoenzymes. This will lead to a decrease in the biotransformation of the drug.[1]

It is hypothesized that VCH is ovotoxic to mice and not rats, because rats are less capable of transforming VCH to the more destructive VCH-1,2-epoxide via cytochrome P450 enzymes. Female B6C3F1 and 129/J mice (age not given) exhibit a 4- to 6-fold greater rate of epoxidation of VCH (1mM) to the ovotoxic VCH-1,2-epoxide as compared to Fischer 344 rats. Approximately 80% of this conversion is due to the actions of the P450 IIA3 and P450 IIB9 isoforms of the CYP enzymes. Since these enzymes are constitutively expressed in the mouse
liver (but not the rat), the mouse is more susceptible to oocyte destruction stemming from VCH administration. VCH is epoxidized by either one or both of the P450 IIA3 isoforms in the mouse, but there is no homologous isoform of this CYP in the rat. Female Fischer 344 rats (age not given) exhibit much lower levels of P450IIB isoenzyme per milligram of body weight, so they are not able to efficiently convert VCH to its ovotoxic epoxide form. Since VCH can be epoxidized when rats are administered phenobarbitol (which induces P450 IIB), there is evidence that other P450 enzymes are involved in VCH epoxidation in the rat. In addition, although VCH is epoxidized in these phenobarbitol-treated rats, the VCH-1,2-epoxide does not appear in the blood, suggesting that the VCH-1,2-epoxide is rapidly degraded by the rat. It is possible that VCH epoxidation is also carried out directly in the ovary.\[^2\]

When female B6C3F1 mice (43-60 days old) are treated with VCH (7.5 mmol/kg/d, ip) for 10 days, there is an increase in the rate of conversion of VCH to VCH-1,2-eposide and VCH-7,8-epoxide. The conversion of VCH-1,2-epoxide to VCD is also increased. This demonstrates that VCH is able to induce its own metabolism. Microsomal P-450 levels are increased by 45 to 65%, compared to untreated control mice. VCH administration induces CYP2A, CYP2B, and CYP2E1. GST activity is also increased after 5 days of VCH treatment. After 5 and 10 days of VCH treatment, blood levels of VCH-1,2-epoxide are increased, and after 10 days blood levels of VCD are elevated. These increased levels fall after day 15 of treatment. These increased levels of VCH-1,2-epoxide and VCD are not due to accumulation of these epoxides in the blood. CYP2A, CYP2B, and CYP2E1 may all be involved in the metabolism of VCH-1,2-epoxide to VCD. VCD is formed in mice after a single dose of VCH that is known to be capable of depleting 85% of primordial follicles in mice. When VCH is repeatedly administered to mice, there is an increase in the activity of the detoxifying enzymes epoxide hydrolase and cytosolic
GST. It is possible that these detoxifying enzymes are attenuating the increase in VCH epoxide products and reducing the levels of these epoxide products by day 15 of VCH treatment.[4]

When hepatic microsomes from female B6C3F1 mice (28-38 days old) and Fischer 344 rats (28-38 days old) are exposed to VCH (1mM), the mouse hepatic microsomes form more VCH-1,2-epoxide and VCH-7,8-epoxide than the rat hepatic microsomes. Pretreatment of rats and mice with acetone (an inducer of CYP2A, CYP2B, and CYP2E1) causes an increase in the formation of VCH-1,2-epoxide in both mice and rats, and an increase in the formation of VCD in mice. Since 5 and 10 day VCH pretreatment does not increase levels of CYP2E1 in mice or rats, and CYP2E1 knockout mice do not exhibit different VCH epoxidation rates than control mice, it is suspected that CYP2E1 is not involved in the epoxidation of VCH.[5]

VCH exists as a racemic mixture of two enantiomers: (R)-VCH and (S)-VCH. (R)-VCH forms more than twice the amount of VCH-1,2-epoxide as (S)-VCH does, but less VCH-7,8-epoxide. Neither enantiomer forms VCD in mouse hepatic microsomes. Since VCH-1,2-epoxide is the major epoxide product, more (R)-VCH is converted to monoepoxide than (S)-VCH. When female B6C3F1 mice (28-38 days old) are pretreated with phenobarbitol or acetone, (R)-VCH (1mM) preferentially forms VCD. When mice are pretreated with VCH, (R)-VCH forms as much VCD as (S)-VCH (1mM). This could mean that other CYP isoforms are induced by VCH to form VCD from (S)-VCH. Rat hepatic microsomes convert (R)-VCH to twice as much VCH-1,2-epoxide as (S)-VCH, but at lower levels as compared to mice. When female Fischer 344 rats (28-38 days old) are pretreated with phenobarbitol, acetone, or VCH no VCD is formed in vitro.[6]

Untreated mouse hepatic microsomes form more VCH-1,2-epoxide and VCH-7,8-epoxide than untreated rat hepatic microsomes. Untreated mouse and rat hepatic microsomes are
unable to form VCD in vitro. Pretreatment of female B6C3F1 mice (28-38 days old) with VCH (1mM) results in a 3.8-fold increase in VCH-1,2-epoxide formation, a 2.0-fold increase in VCH-7,8-epoxide formation, and the formation of VCD. Pretreatment of female Fischer 344 rats (28-38 days old) with VCH (1mM) results in a 2.0-fold increase in VCH-1,2-epoxide formation and a 1.8-fold increase in VCH-7,8-epoxide formation, but no VCD formation. Only in mice does repeated exposure to VCH or VCH-1,2-epoxide lead to an increase in total hepatic CYP levels. Treatment of mouse hepatic microsomes with VCH or VCH-1,2-epoxide results in an increase in CYP2A and CYP2B protein levels. Treatment of rat hepatic microsomes with VCD causes an increase in CYP1A and CYP2C protein levels. The activity of the CYP1A, CYP2A, and CYP2B isoforms are increased in the mouse with exposure to VCH or VCH-1,2-epoxide. Repeated VCH, VCH-1,2-epoxide, or VCD administration did not change the activity of the CYP2E1 isoform in either the mouse or rat. Since pretreatment with VCH-1,2-epoxide results in higher total CYP levels but lower CYP2C6, CYP3A2, CYP2A6, CYP2B1, CYP2E1, CYP1A1, and CYP3A1 levels than VCH treatment, it is suspected that other CYP isoforms play a role in VCH and/or VCH-1,2-epoxide biotransformation. In human hepatic microsomes, the CYP2B6 and CYP2E1 isoforms preferentially form VCH-7,8-epoxide and VCH-1,2-epoxide, respectively. VCD was formed from the VCH monoepoxides by CYP2A6, CYP2B6, CYP2E1, and CYP3A4.[7]

There are eight enantiomeric VCD products that can be formed from the epoxidation of the three VCH monoepoxides. When CYP2B1 is induced in male Sprague Dawley rat (age not given) hepatic microsomes, there is an increase in the yield of the diepoxide products of the epoxidation of VCH monoepoxides. When CYP2E1 or CYP3A1/2 are induced, the yield of VCD is similar to the yield of control rat hepatic microsomes, and only certain enantiomers of
the diepoxides are formed. This means that the product yield and the stereoselectivity of the epoxidation process are dependent upon the P450 isoenzyme induced. The monoepoxide (1R,2S,4R)-trans-3b enantiomer accumulates in the rat and the mouse. When this enantiomer is epoxidized it forms almost exclusively the (1R,2S,4R,7R)-trans-10b diepoxide. This occurs in untreated rat hepatic microsomes, phenobarbitol treated rat hepatic microsomes, and P4502B1 induced rat hepatic microsomes. CYP2E1 is incapable of performing the (1R,2S,4R)-trans-3b to (1R,2S,4R,7R)-trans-10b diepoxide reaction. All eight diepoxide enantiomers act as substrates for mEH. When VCH is epoxidized in a P450-induced environment, the enantiomer (1R,2S,4R,7R)-trans-10b diepoxide is the primary product. In addition, the (1R,2S,4R,7R)-trans-10b diepoxide isomer does not readily undergo mEH catalyzed hydrolysis. This indicates that the (1R,2S,4R,7R)-trans-10b diepoxide is the likely isomer responsible for the mutagenic and carcinogenic properties of VCH. VCH is primarily oxidized to (1R,2S,4R)-trans-3, which is almost solely epoxidized to (1R,2S,4R,7R)-trans-10. It is possible that this enantiomer’s particular configuration is related to its ability to cross-link, which is the source of a diepoxide’s mutagenic capabilities.[9]

*Detoxification of VCH and VCD by Ovarian Cytochrome P450 Enzymes*

After 15 days of administration of VCH (7.4 mmol/kg/d, ip) or VCD (0.57 mmol/kg/d, ip) to female B6C3F1 mice (28 days old), mRNA encoding for ovarian cytochromes CYP2E1 and CYP2A are increased in small preantral follicles (25-100 μm; those follicles specifically targeted by VCD). mRNA encoding for CYP2E1 is increased in antral follicles following VCD administration. mRNA encoding CYP2A is increased in interstitial cells following VCH dosing. mRNA encoding for CYP2B is increased in small preantral follicles after 15 days of VCH
dosing. After repeated dosing with VCH, CYP2E1 protein is reduced in interstitial cells, CYP2A protein is increased in interstitial cells, CYP2B protein is increased in the granulosa cells of small primary follicles, and CYP2B protein is increased in granulosa cells of antral follicles. After repeated dosing with VCD, CYP2B protein is increased in granulosa cells of antral follicles. After repeated VCH exposure, CYP2E1 activity is increased and CYP2B activity is unchanged, while CYP2A activity cannot be detected. CYP2E1 exhibited a higher baseline activity towards a model substrate than either CYP2A or CYP2B. In addition, VCH caused an increase in CYP2E1 activity. This implies that CYP2E1 has a role in ovarian metabolism of VCH. Interstitial cells exhibited high levels of CYP2E1, CYP2A, and CYP2B protein, which could indicate that the interstitial cells are involved in epoxidizing VCH. Since the VCH- and VCD-vulnerable small preantral follicles exhibit an increase in mRNA for CYP2E1, CYP2A, and CYP2B after repeated VCH or VCD dosing, it is possible that VCH is bioactivated by these isoforms in the ovary.[10]

In female CYP2E1 +/+ wild-type mice (28 days old), primordial follicle loss occurs at lower concentrations of VCH-1,2-epoxide compared to control mice, more unhealthy primordial and primary follicles appear with 1000 μM VCH-1,2-epoxide injections compared to control mice, primordial follicle loss occurs with VCH administration, and ovaries tend to be more susceptible to VCD as compared to control mice. When CYP2E1 is not present in mouse ovaries, VCH-1,2-epoxide will not cause loss of primordial follicles. Thus, ovarian CYP2E1 is necessary for the conversion of VCH-1,2-epoxide to VCD. Since cultured ovaries are more sensitive to VCD than VCH-1,2-epoxide, it is likely that the ovarian biotransformation of VCH-1,2-epoxide to VCD is hindered by mEH detoxification, is an inefficient conversion, or is detoxified by glutathione conjugation. Although ovarian CYP2E1 can convert VCH-1,2-epoxide
to VCD, it is not required for this conversion to occur in vivo. Therefore it is likely that hepatic biotransformation of VCH-1,2-epoxide to VCD plays the largest role in this conversion, and that the ovarian biotransformation plays only a small role.[11]

Ovarian microsomal expoxide hydrolase (mEH) is involved in metabolism and detoxification of xenobiotics, such as VCH and VCD. Immature (28 days old) B6C3F1 mice dosed with VCH (7.4 mmol/kg/d, ip) for 15 days display increased expression of mEH mRNA in small preantral (25-100 μm) and large preantral (100-250 μm) follicles as compared to controls. Repeated VCH treatment decreases mEH expression in interstitial cells. Immature mice dosed with VCD (0.57 mmol/kg/d, ip) for 15 days display increased mEH expression in small preantral, large preantral, and antral (>250 μm) follicles as compared to controls. Repeated VCD treatment does not affect mEH expression interstitial cells. In control mice, mRNA expression of mEH is highest in antral follicles. Repeated daily dosing with either VCH or VCD increases mEH activity levels in small preantral follicles. There is also a nonsignificant trend for the mEH activity of large antral and interstitial cells to increase. In control mice, mEH activity levels are highest among growing preantral follicles. mEH protein is evident in oocytes, granulosa cells, theca cells, and, most strongly, interstitial cells. VCH or VCD dosing increases mEH protein expression in the theca cells of growing preantral follicles. Since theca cells may act as a protective barrier against blood-born toxins, small preantral follicles that lack theca cells may be more susceptible to destruction by VCH or VCD. This data provides further evidence that preantral follicles induce production of mEH as a protective response against VCH or VCD exposure.[40]

**Detoxification of VCH and VCD by Glutathione**
Glutathione (GSH) is an antioxidant that protects cells against oxidative stress and electrophilic compounds. GSH can either conjugate to electrophilic compounds in order to detoxify them or can directly reduce them through the formation of an oxidized dimer (GSSG). The possible by-products of GSH-VCD conjugation have been observed in female Fischer rat and B6C3F1 mouse urine. One dose of VCD (0.57 mmol/kg) reduces female Fischer rat (21 days old) liver GSH content by 48% after 2 hours. The GSH levels return to normal after 6 hours. Ovarian GSH content is not affected by a single dose of VCD. When rats are dosed repeatedly with VCD for 15 days, GSH levels are reduced by 2 hours following the last VCD dosage, but return to normal 26 hours after the last dose. The reduction in GSH levels is greater after a single dose of VCD than they are after repeated doses. Additionally, since no increases in GSSG were seen, it is likely that GSH conjugates with VCD in order to detoxify it for excretion. Since 70% of a single dose of VCD is excreted by rats 6 hours after administration, it is expected that GSH levels will spike and fall quickly.\[8\]

VCH, VCH monoepoxides and VCD all deplete hepatic GSH when administered to male albino Swiss mice (age not given). Administration of VCH, VCH monoepoxide, and VCD cause a fall in male albino Swiss mice hepatic GSH levels. VCH depletes mice hepatic GSH at slower rates than VCH monoepoxide or VCD. VCH and VCH monoepoxides induces aminophyrine-N-demethylase activity and increase NADPH cytochrome c reductase levels (VCH monoepoxides especially). Cytochrome P-450 is induced by VCH monoepoxides, but not VCH or VCD. VCH monoepoxides slightly induces the detoxifying epoxide hydrolase.\[12\]

**Ovarian Effects of VCH**
Ovaries that are devoid of oocytes secrete more gonadotropin, which promotes the development of ovarian tumors. VCH administered to female B6C3F₁ mice (28 days old) destroys small oocytes in a dose-dependent fashion but does not change the number of small oocytes in rats. The ED₅₀ values of VCH are 4-, 5-, and 14-fold higher than the ED₅₀ for VCH-7,8-epoxide, VCH-1,2-epoxide, and VCD respectively. It was only after 15 days of VCH administration (800 mg/kg, ip) that small oocytes were reduced in mice ovaries. Increasing doses of VCH (100, 400, and 800 mg/kg, ip) resulted in higher blood concentrations of VCH-1,2-epoxide. The ED₅₀ dose of VCH resulted in high blood concentrations of VCH-1,2-epoxide between 30 minutes to 2 hours after VCH administration, while the ED₅₀ dose of VCH-1,2-epoxide resulted in high blood concentrations of VCH-1,2-epoxide between 5 and 15 minutes after VCH-1,2-epoxide administration. Chloramphenicol, a known inhibitor of CYP450 metabolism, administered to mice results in a 69% reduction of VCH epoxidation. Thus, inhibition of VCH epoxidation reduces the ovarian toxicity of VCH. These results suggest that ovarian neoplasms are related to VCH-induced oocyte destruction. They also suggest that VCH epoxides are more potent inducers of ovarian toxicity than VCH and that VCH epoxidation is a key step in VCH-induced ovarian toxicity.⁹⁸

Ethylcyclohexene (ECHE) and cyclohexene (CHE) are analogues of VCH that contain an unsaturated site that mimics the VCH-1,2-epoxide. Vinylcyclohexane (VCHA) contains an unsaturated site that mimics VCH-7,8-epoxide. Female B6C3F₁ mice (28 days old) treated with the VCH analogues ECHE, VCHA, and CHE did not exhibit any form of follicle depletion. In addition, the monoepoxides of these analogues did not deplete mouse ovarian follicles. Isoprene (ISO), butadiene monoepoxide (BMO), and butadiene diepoxide (BDE) are either diepoxides or can be metabolized to diepoxides. Mice treated with ISO, BMO, and BDE exhibited ovarian
follicle depletion similar to that observed after VCH administration. These results indicate that VCH must be epoxidized at both of its unsaturate sites to be ovotoxic. When mouse hepatic microsomes were exposed to ECHE, VCHA, and CHE, they were able to epoxidize them to their monoepoxide forms at rates similar to the epoxidation rate of VCH. After administration of a single dose of ECHE, VCHA, and CHE, the monoepoxides of these analogues were observed in the blood. In activity studies, the VCH analogue monoepoxides and VCH-1,2-epoxide were able to alkylate nicotinamide at similar rates. However, VCD was able to alkylate nicotinamide at a rate that was 3.5-fold greater. BDE was able to alkylate nicotinamide at a rate 3.5- to 10-fold higher than the monoepoxides. This rate was 3.8-fold higher than the VCD alkylation rate, which is logical since BDE was ovotoxic at a 4-fold lower dose than VCD. Taken together, these results indicate that VCD is the ovotoxic metabolite of VCH.[39]

Ethylcyclohexene (ECHE) and cyclohexene (CHE) are analogues of VCH that have one unsaturated site that corresponds to VCH-1,2-epoxide. Vinylcyclohexane (VCHA) is a VCH analogue that has one unsaturated site that corresponds to VCH-7,8-epoxide. Although all three analogues are metabolized to monoepoxides in female B6C3F1 mouse (46 days old) hepatic microsomes at the same rate VCH is epoxidized, none of these analogues are ovotoxic like VCH. The monoepoxides of the VCH analogues were also observed at circulating levels in the blood. This is further evidence that VCH must be epoxidized at both unsaturated sites in order to exhibit ovotoxicity.[41]

The conversion of VCH to VCH-1,2-epoxide occurs in female Crl: CD BR rat (42-71 days old) and B6C3F1 mouse (approximately 72 days old) liver and lung. Mouse liver exhibited a maximum enzyme velocity that was 56-fold higher than rat liver. The lungs of both species had higher maximum enzyme velocities than the liver of the same rodent, with mouse lung
exhibiting a maximum enzyme velocity that was 2.5- to 3.8-fold higher. The conversion of VCH to VCH-7,8-epoxide occurs in rat and mouse liver, but only in mouse lung. However, only the mouse can produce the VCH-7,8-epoxide with any efficiency. In addition, the mouse produces the VCH-7,8-epoxide at lower rates than it does the VCH-1,2-epoxide. Neither reaction occurs in the ovaries of either rats or mice. VCD was also not produced by mouse or rat ovaries. VCD was produced from VCH-1,2-epoxide and VCH-7,8-epoxide at similar rates in the mouse and rat liver. VCD was also produced in the lung, with mice converting VCH-1,2-epoxide or VCH-7,8-epoxide to VCD at greater rates than the rat. VCH-1,2-epoxide was metabolized to VCH-1,2-diol by rat and mouse liver at similar rates. Rats were able to hydrolyze VCD in the liver lung and ovary, while mice were able to hydrolyze VCD in the liver and lung. The metabolism of the VCH-1,2-epoxide to VCD occurred with more efficiency than the metabolism of VCH to VCH-1,2-epoxide. Overall, the mouse produces VCH monoepoxides more efficiently and hydrolysis of the monoepoxides is more efficient in rats. This leaves the mouse more susceptible to ovotoxicity induced by VCH.

Differences Between Mice and Rats in Susceptibility to VCH Toxicity

Twenty-four hours after administration of a single oral dose of radioactively labeled VCH (400 mg/kg), female B6C3F1 mice (17-23 grams) had eliminated 97% of the VCH while rats had eliminated 88% of the VCH. 48 hours after administration, 100% of the VCH had been eliminated by female Fischer 344 rats (175-250 grams). 50-60% of the VCH was eliminated through urine by both mice and rats. Approximately one-third of the VCH was eliminated through expiration in both rats and mice. No tissue in the mouse contained more than 1% of the VCH dose after 24 hours. Adipose tissue, muscle, and skin of rats contained 3.4, 1.1, and 1.1%
of the VCH dose respectively after 24 hours. The concentration of VCH in the ovary per milligram of tissue was equivalent to the amount observed in the livers of both mice and rats. VCH concentrations were highest in adipose tissue of rats and mice, which is logical due its lipophilic nature. VCH concentrations were highest in mouse adipose tissue between 1 and 2 hours after VCH administrations, while they were highest in rat adipose tissue 8 hours after VCH administration. Although mice exhibited VCH-1,2-epoxide in their blood up to 6 hours after administration, the highest levels of VCH-1,2-epoxide were seen two hours after VCH administration. Rats did not display measurable amounts of VCH-1,2-epoxide, and VCH-7,8-epoxide could not be detected in either mice nor rats. Mouse hepatic microsomes epoxidized VCH at a rate 6.5-fold greater than rat hepatic microsomes, resulting in the production of 4 times more VCH-1,2-epoxide. Since the difference in blood concentrations of VCH-1,2-epoxide was more than 6.5-fold greater between mice and rats, it is assumed that there must also be a difference in VCH-1,2-epoxide detoxification rates.\[37\]

After a single dose of VCD (100 mg/kg, ip) female B6C3F1 mice (19-22 grams) excreted 80% of the VCD dose through urine while female Fischer rats (150-170 grams) excreted 90% of the dose through urine, 72 hours after VCD administration. Rats excreted the VCD faster than mice. The major metabolite in rat urine was 4-(1,2-dihydroxy) ethyl 1,2-dihydroxycyclohexane, or the tetrol product. The major metabolite in mouse urine was VCD. By 72 hours after the administration of VCD, less than 1% of the dose was contained in tissues of mice and rats. The liver, spleen, and kidney of the mice and rats contained the highest amount of VCD. After a single bolus dose of VCD (10 mg/kg, iv), VCD was quickly eliminated from the blood plasma. The tetrol metabolite of VCD remained in the blood for up to an hour. Mice cleared the VCD from the plasma faster but had 5-fold greater volumes of distribution into which VCD could be
distributed. By 6 hours after VCD administration, 17% of the dose is excreted in bile in rats. When hepatic GSH is depleted, there is much less VCD excreted in the bile. The majority of the VCD excreted in bile was present as polar conjugates. The liver, kidney, and brain exhibited the highest concentrations of VCD following the intravenous dose in rats. The maximum enzyme velocity for hepatic microsomal conversion of VCD to its tetrol metabolite was 4 times more rapid in the rat. After a single dose of VCD (80 mg/kg, ip) hepatic and ovarian GSH levels were decreased faster and for longer periods of time in the mouse. By 24 hours after the VCD administration, the GSH levels had returned to normal in both the mouse and rat. GSSG levels were never altered. This indicates that mice form VCD-GSH conjugates more than rats.\textsuperscript{[43]}

**Ovarian Effects of VCD**

The ovariectomized model of menopause does not result in a period of perimenopause and there is no residual ovarian tissue left in the animal. Other models of menopause are also problematic. For example, transgenic mice used as a model of menopause have their reproductive systems affected during fetal development. In addition, nonhuman primates undergo ovarian failure late in their lives. Furthermore, the haploinsufficient FSH receptor (±) mouse undergoes perimenopause from 3 to 12 months of age and therefore develop compromised ovarian function and uterine pathologies. The VCD model does not exhibit any of these limitations.\textsuperscript{[54]}

**Follicle Changes Induced by VCD**

Female Fischer rats (21 or 51 days old) administered VCD (80 mg/kg/d, ip, 30 d) display uterine weights that are approximately 25% less than those displayed by controls. Rats that are
immature (21 d) at the onset of VCD dosing exhibit no change in the number of regular estrous cycles over 30 days. However, rats that are adult (51 d) at the onset of VCD dosing exhibit a 40% reduction in the number of regular estrous cycles per 30 days. On day 31 of dosing, ovaries isolated from VCD treated adult rats exhibit a 33-38% reduction in primordial and primary follicle counts, while ovaries isolated from VCD treated immature rats exhibit a 19-45% reduction. Only in immature rats are the numbers of growing follicle reduced (by 54% compared to controls). This likely occurs because the ovarian follicles of immature rats have not yet matured to the growing stage before VCD begins to exert effects on the follicles. Only adult rats exhibited decreased uterine weights, which likely resulted from the decreased number of regular estrous cycles leading to lower estrogen levels and less uterotrophic effects.[60]

![Image of ovaries](image)

Fig. 1.12 Micrographs of B6C3F1 mouse ovaries depicting ovarian atrophy following VCD treatment. The ovaries of immature (21 day old) sesame oil vehicle controls (A) and mice treated with VCD (160 mg/kg/d, ip) for 15 days (B) are shown 120 days post-treatment.[58]

A single high dose of VCD (320 mg/kg, ip) resulted in a time-dependent decrease in primordial and small primary follicles of 28 day old female Fischer 344 rats beginning 6 days
after administration. After 36 days, the ovaries from rats administered this single high dose exhibited reduced follicle counts in all except the large primary follicles.\textsuperscript{[63]}

Female Fischer 344 rats (21 days old) administered one dose of VCD (80 mg/kg, ip) exhibited no difference in ovarian follicle counts from control rats 24 hours after receiving VCD. After 15 days, the number of primary follicles was 41% greater than control rats. The normal rate of atresia in control rats results in significant decreases in large primary follicle counts between 28 and 43 days of age (analogous to day 1 through 15 of VCD treatment). Twenty-four hours after the single dose of VCD, there were 30% more healthy large primary follicles in the ovaries of rats treated with VCD. Twenty-four hours and 15 days after VCD administration, there were more healthy primary follicles compared to controls. This can be compared to 15 daily doses of VCD, wherein the number of healthy primordial, small primary, and large primary follicles are reduced compared to controls. Four hours after the single VCD dose, levels of mRNA encoding \textit{bax} were reduced by 44% and Bax protein was observed in the cytoplasm of oocytes and granulose cells for some primordial, primary, and growing follicles. After 15 days, the mRNA levels return to control levels. This can be contrasted to 15 daily doses of VCD, wherein the levels of mRNA encoding \textit{bax} are increased by 229%. This implies that a single dose of VCD is protective because it not only results in greater numbers of healthy primary follicles (without reducing the total number of primary follicles) but it reduces the expression of the atresia-inducing \textit{bax}.\textsuperscript{[52]}
Female Fischer 344 rats (28 days old) dosed with VCD (80 mg/kg, ip) for 6, 8, 10, 12 or 15 days exhibited significant decreases in primordial and primary follicles after 12 days. Female B6C3F1 mice (28 days old) dosed with VCD (80 mg/kg, ip) for 6, 8, 10 or 12 days exhibited significant decreases in primordial and primary follicles after 12 days, at levels greater than those seen in rats. Primordial and primary follicles in both rats and mice displayed evidence of atresia at day 12. In rats, an increase in atretic primordial follicles appeared on day 12 and an increase in atretic primary follicles appeared on day 10. In mice, an increase in atretic primordial follicles appeared on day 8 and an increase in atretic primary follicles appeared on day 12. In mice and rats follicle damage, as evidenced by the appearance of atretic follicles, preceded follicle loss by
at least 2 days. Since primordial follicle damage occurs before primary follicle damage and primary follicle loss occurs at a steady rate (as evidenced in prior research), this implies that VCD initially targets primordial follicles. In addition, mice appeared more susceptible to VCD administration by exhibiting earlier follicle damage and more extensive follicle loss than rats.[53]

**Hormonal Changes Accompanying VCD Treatment**

In mice, ten days of persistent diestrus coinciding with no observable ovarian follicles is indicative of ovarian failure. Female B6C3F1 mice (28 days old) dosed with VCD (160 mg/kg/d, ip) for 10 days exhibited ovarian failure 104 to 166 days after dosing while mice dosed for 20 days exhibited ovarian failure 43 to 63 days after dosing. In mice dosed for 10 days FSH levels increased beginning on day 44, while FSH levels began increasing on day 35 in animals dosed for 20 days. In mice dosed for 10 days, levels of primordial, small primary, and large primary follicles were reduced. In mice dosed for 20 days, levels of primordial, small primary, large primary, secondary, and antral follicles were reduced. VCD administration resulted in decreased ovarian and uterine weights as compared to control, which was determined to be a result of ovarian failure and not a direct effect of VCD. The longer duration of VCD administration resulted in earlier onset of ovarian failure. Since FSH levels rose and remained elevated at similar timepoints in both experimental groups but the two groups did not undergo ovarian failure at the same time this implies that the timing of FSH increase cannot predict the onset of ovarian failure in mice.[54]

Immature (28 d) Fischer 344 rats administered VCD (80 mg/kg, ip) for 30 days exhibit reduced primordial and primary follicle counts by day 30, reduced secondary follicle counts by day 60, and reduced antral follicle counts by day 120. Compared to control rats (sesame oil, ip)
primordial, large preantral, and antral follicles of VCD dosed rats appeared to be undergoing apoptosis by a similar manner. In the large preantral and antral follicles the oocyte and granulosa cells lost focal contact before the granulose cells underwent apoptosis and the oocyte was lost. In the primordial follicles the oocyte accumulated vacuoles while the granulosa cells underwent no changes. In VCD rats, plasma FSH levels increase beginning on day 120 and are inversely correlated with loss of antral follicles. Plasma levels of 17β-estradiol remain similar to control rats until day 360, when they become variable among individual VCD-dosed rats. Up to day 360, VCD-dosed rats exhibit greater numbers of days in proestrus and estrus, which is explained by the change in hormone levels observed. When acyclicity is achieved FSH levels plateau and 17β-estradiol levels decrease. By day 360, VCD-dosed rats exhibit no ovarian follicles as evidenced by morphologic analysis. In control rats, primordial follicle counts are reduced beginning on day 240. VCD treated rats exhibit reduced ovarian weights compared to controls, which is explained by the loss of cells that produce 17β-estradiol. This in turn eliminates the negative feedback mechanism on the pituitary so that FSH levels increase, disrupting cyclicity.\[55\]

Preovulatory follicles and copora lutea secrete 17β-estradiol (E2) and progesterone (P4) respectively. These steroids act through a negative feedback mechanism on the hypothalamus and anterior pituitary to decrease follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Inhibins produced by the ovary also act to decrease FSH secretion from the pituitary. With the loss of large antral follicles and corpora lutea E2, P4, and inhibin levels are decreased and FSH and LH levels rise. A cycle length is defined as the time between two successive demonstrations of proestrus. Immature B6C3F1 mice (28 days old) dosed with VCD (160 mg/kg, ip) for 15 days exhibit longer cycles than control mice beginning in cycle 5 (8.5 d versus
5.1 d, respectively). As cycles lengthened in VCD mice days in proestrus decreased. In cycles 9 and 11, progesterone (P4) is higher in VCD treated mice, but this was not part of a trend and appears to have been the result of individual animal variability. Furthermore, since the E2:P4 ratio remained the same between VCD-dosed and control rats over 12 cycles it would appear that the production of E2 and P4 were similar between VCD and control rats. During cycles 5 and 12, VCD-dosed mice exhibited an increase in plasma FSH levels. This increase was concurrent with the onset of irregular cycle length. VCD-treated mice exhibited lower ovarian weights (atrophy caused by ovarian failure) and no ovarian follicles after 12 cycles. Ovarian failure occurred on average at 85±6 days after VCD dosing. The time of proestrus is analogous to the preovulatory peak in E2 in humans, so blood samples were taken in mice at this time. It is evident that the mice exhibit the same onset of irregular cycles, irregular E2 changes, and FSH increases that women undergo during perimenopause.[56]

Even with follicle loss, ovaries are still capable of producing androgens because the theca-derived interstitial cells that produce them remain intact. Interstitial ovarian cells contain lipids, LH receptors, HDL receptors (SR-BI), and steroidogenic enzymes that induce the production of androgens. The theca cells of atretic follicles produce the interstitial cells. The small preantral follicles isolated from female B6C3F1 mice (28 days old) administered VCD (160 mg/kg/d, ip) for 10 days exhibit an increase in caspase-3 cleavage activity. VCD treated mice exhibited irregular cycles (those falling outside the mean cycle length of control animals) beginning on day 58 after VCD dosing (29%). By day 37 post-VCD (160 mg/kg/d, ip, 15 d) administration half of all mice exhibited irregular cycles. Forty-six days after treatment 75% of the VCD treated mice exhibited irregular cycles, with all mice showing irregular cycles by day 58. Plasma FSH levels were increased in VCD-dosed mice 37 days after treatment but reached a
plateau by day 100. Between days 15 and 46 the levels of circulating FSH were inversely related
to the number of antral follicles in the ovaries of VCD treated mice. Although liver weights
were modestly increased on days 15 and 37, hepatic enzyme levels fell within normal range
indicating no impaired liver function in VCD mice. Ovarian atrophy occurred 120 days after
VCD treatment. By day 127 plasma LH and FSH levels were increased in VCD mice relative to
controls, while plasma levels of progesterone and androstenedione were reduced, and levels of
17β-estradiol fell to non-detectable levels. This leaves the ratio of androgens to estrogen higher
than control mice, indicating that the ovaries of VCD-treated mice are relatively rich in
androgens. Mice treated with VCD exhibited a more diffuse distribution of LH receptor in
residual ovarian tissue. They also exhibited a more uniformly dispersed distribution of SR-BI,
which indicates active cellular steroidogenesis. In immunofluorescent staining, the SR-BI of
VCD treated mice stains at a lower intensity than those of control mice. This indicates that the
residual ovarian tissue of VCD-treated mice is similar to interstitial cells. Ovarian cells removed
on day 37 of treatment in both VCD (160 mg/kg/d, ip, 15 d) and control mice produce
progesterone upon LH stimulation on day 120 of cell culture. However, the ovarian cells
isolated from VCD treated mice produce 20-40% less progesterone than those of controls. The
ovarian cells of mice administered VCD exhibited significantly less 17β-estradiol compared to
controls. While the ovarian cells of control mice produce androstenedione at levels lower than
the limit of detection, VCD-treated animals produce detectable levels of androstenedione.
Insulin stimulates adrostenedione production in VCD ovarian cells at concentrations of 1 and 3
ng/mL. This data suggests that circulating androstenedione levels are produced by the ovary,
especially considering that the rat adrenal glands do not produce androgens.\[58\]
Fig. 1.14 Comparison of VCD-induced ovarian failure in mice and menopause in humans. As compared to
ovariectomized mice, VCD-treated mice more closely mimic what occurs in the human peri-menopausal transition
in terms of estrogen levels, cyclicity, and FSH levels. The peri-menopausal transition occurs approximately 1-10
years prior to menopause and the cessation of menstrual cycle.\textsuperscript{[23]}

\textit{Ovarian Effects of VCD in Sprague Dawley Rats}

A majority of the studies that have examined the ovotoxic effects of VCD in rats have
utilized the Fischer strain of rats. Very few studies have examined the ovotoxic effects of VCD
in Sprague Dawley rats, which is the rat strain most commonly utilized for osteoporotic bone
studies employing the ovariectomized model. Female Sprague Dawley rats (129 ± 4.8 days old)
anesthetized and administered VCD (80 and 160 mg/kg/d) for 10 to 15 days intramuscularly in
the semimembranosis/biceps femoris muscle exhibited faster and more efficient primordial
follicle destruction than rats given VCD through intraperitoneal administration. Rats
administered 160 mg/kg/d lost significant amounts of weight and at necropsy exhibited severe
necrotizing myositis and intralesional foreign material at the injection site. Rats administered 80
mg/kg/d exhibited only a moderate focal fasciitis, myositis, and steatites with necrosis at the
injection site. Rats administered both doses exhibited reduced primordial follicle counts
compared to controls. The rats given 80 mg/kg/d (im) displayed an 80% loss in primordial
follicles with 15 days of VCD administration, while previous studies indicate that rats
administered the same dose intraperitoneal display a 70% reduction in primordial follicles with
30 days of dosing.[50]

Female Sprague Dawley rats administered VCD (160 mg/kg/d, ip, 20 d) at 28 days of age
exhibit irregular estrous cycles as follicle depletion occurs. Estrous cycle lengths increase as
days spent in estrus increase. By the time the rats exhibit irregular cycles, follicle counts of their
ovaries indicate that they are almost completely follicle deplete. Within 8 months of the initial
VCD dosage (160 mg/kg/d, ip, 20 d) the rats are completely follicle deplete. The follicle deplete
ovaries of VCD rats are smaller than controls (1-2mm versus 3-4 mm), and exhibit more
superficial fat than control ovaries.[59]

**Apoptosis of Ovarian Follicles in Response to VCD**

Apoptosis is a controlled process whereby unwanted or potentially harmful cells are
eliminated from tissues. This is in contrast to necrosis, which is the passive response whereby
injured cells die as a result of trauma. To identify whether apoptosis or necrosis is occurring,
morphological criteria are employed. Apoptosis is identified by means of nuclear pyknosis,
cytoplasmic condensation, and loss of focal contact between adjacent cells. Necrosis is
identified by means of macrophage infiltration, membrane rupture, and swelling. In 28 day old
female Fischer rats dosed with VCD (80 mg/kg) for 6, 8, 10, 12, or 14 days and their ovaries
were removed one hour after the final dosage. In rats dosed for 10, 12, or 14 days, there was an increase in DNA degradation as compared to controls. In rats dosed in the same manner, whose ovaries were collected 24 hours after the final dosage, there was no evidence of DNA degradation. In rats dosed in the same manner, whose ovaries were collected 4 hours after the final dosage, there was an increase in VCD-induced low-molecular-weight DNA fragmentation, for rats dosed for 10 and 12 days. This means that day 10 of dosing is the earliest time point at which impending follicular destruction can be observed. This also means that damaged cellular DNA is rapidly cleaned up by surrounding tissue. However, rats dosed with VCD (80 mg/kg, ip) for 8, 10, or 15 days only exhibited primordial and primary follicle loss after 15 days of VCD treatment. The ovaries collected 4 hours after dosing from rats dosed for 10 days exhibited irregularly shaped primordial and primary follicles that had margination of chromatin along the nuclear membrane. The granulosa cells were not uniform in shape, were irregularly arranged around the oocyte, and had no focal contact with the oocyte. Some granulosa cells also had margination of chromatin along the nuclear membrane. These signs are all characteristic of apoptosis, and not necrosis. At no time point did the DNA degradation that occurred mimic the laddering pattern normally seen with apoptosis, but this can be explained by the fact that rat ovaries lack the necessary endonucleases that cleave internucleosomal DNA.[26]

Apoptosis is associated with an increase in the expression of members of the \textit{bcl-2} gene family, one of which is \textit{bax}. It is also controlled by increases in antioxidant enzymes that are a reaction to increases in reactive oxygen species by toxic compounds. Three such antioxidant enzymes are forms of the superoxide dismutase (SOD) enzyme: manganese-containing associated with mitochondria (MnSOD), copper/zinc-containing associated with the cytoplasmic compartment (Cu/ZnSOD), and a secreted form (secSOD). Epoxide hydrolase has also been
proposed as a detoxifying enzyme that increases in response to VCD administration. When VCD (80 mg/kg) is administered to female Fischer rats (28 days old) for 10 days, small (25-100 μm) preantral follicles exhibit a significant increase in \( bax \) mRNA (172±20%), a significant increase in MnSOD mRNA (248±70% of control), and a significant increase in mEH mRNA (352±120%). Large (100-250μm) preantral follicles exhibit a decrease in mEH mRNA, but no changes in \( bax \) mRNA or MnSOD mRNA. These results suggest that \( bax \) mediates VCD-induced apoptosis in small rat preantral follicles. There is also evidence that the small preantral follicles may be attempting to counteract the effects of VCD by increasing antioxidative and detoxifying enzymes like MnSOD and mEH.\(^{[27]}\)

In female Fischer 344 rats, oocytes in primordial follicles are rarely atretic, but can be identified by large amounts of cytoplasmic vacuolization and the loss of identifiable organelles. The granulosa cells that surround atretic primordial oocytes remain unchanged. The oocytes contain mitochondria, Golgi complexes, and large nuclei with nucleolonemata. Oocytes in healthy developing follicles after the primary follicle stage are surrounded by a zona pellucida that is penetrated by microvilli from the oocyte and larger projections from the granulosa cells. The cytoplasm of the oocyte exhibits loosely clustered organelles. There are also mitochondria, rough endoplasmic reticulum, parallel linear structures, and condensed chromatin. In follicles of all sizes, atresia begins with apoptosis of the granulosa cells and loss of focal contact between the granulosa cells and the oocyte. As the granulosa cells become lost, the oocyte begins to undergo atresia with organelles becoming randomly distributed, the RER becoming lost, the mitochondria stain darker, and any microvilli projections become retracted and are lost. In the end stages of atresia, most of the granulosa cells are gone and the oocyte divides into segments. Although apoptosis is occurring in atretic follicles, it appears to only be occurring in the
granulosa cells. Atretic oocytes appear to undergo a process of degeneration that is neither apoptosis nor necrosis.\[^{28}\]

Caspases are cysteine proteases that are involved in apoptosis. They are expressed as procaspases that must be activated through cleavage. Initiator caspases (caspase-2, -8, and –9) become activated by proapoptotic signals that involve the binding of cofactors, and they in turn activate executor caspases (caspase-3, -6, and –7). The executor caspases will cleave certain protein substrates, resulting in collapse of the cell. When female Fischer 344 rats (28 days old) are dosed with VCD (80 mg/kg, ip) for 1 or 15 days, caspase-3-like cleavage activity is increased in small (25-100 μm) follicles after 1 day and 15 days of VCD exposure, with greater increases after 15 days. Expression of 32-kDa procaspase-3 protein in the cytosol of small follicles is decreased after 1 day and increased after 15 days of VCD exposure. Expression of the 17-kDa active caspase-3 subfragment protein is increased after 1 day and 15 days of VCD exposure. Although procaspase-3 is found in the cytosol and the mitochondria of small follicles, it is only active in the cytosol. Caspase-3 staining is increased in granulosa cells, primordial follicles, and small primary follicles after 15 days of VCD administration. This increased staining was observed in the cytosol and not the mitochondria of follicles. Caspase-9-like cleavage activity of small follicles was increased in the mitochondria after 1 day and decreased in the cytosol after 15 days of VCD. Caspase-8-like cleavage activity in the cytosol of small follicles was increased after 15 days of VCD. VCD administration causes an increase in caspase-3-like activity, but only in the primordial and small primary follicles that are destroyed by VCD. Only repeated VCD administration leads to an increase in procaspase-3-like activity. Since a single dose of VCD is protective to rat ovarian follicles, this implies that apoptosis is not initiated by caspase-3 alone. Since repeated dosing with VCD is necessary to increase caspase-8 activity, it may be
involved in VCD-induced apoptosis. Caspase-9 activity was initially increased after a single
dose of VCD, but decreased after repeated dosages, which cannot be definitively explained. In
any case, repeated VCD administration results in the activation of a caspase-3 cascade that may
be regulated by caspase-8 and –9.[29]

The oncogenes Bcl-2 and Bcl-xL act to prevent apoptosis, while Bax and Bak act to
accelerate apoptosis. Non-phosphorylated Bad protein is associated with an increase in
apoptosis, through blockage of Bcl-xL. Cytochrome c is associated with activation of the
caspase-9/Apaf-1 apoptosis pathway. Movement of cytochrome c from the mitochondria to the
cytosol is necessary for mitochondria-dependent apoptosis to occur. Fifteen days of VCD (80
mg/kg, ip) administration to Fischer 344 rats (28 days old) results in an increase in
phosphorylated Bad protein levels in small preantral follicles, an increase in non-phosphorylated
Bad protein levels in small preantral follicles, a decrease in Bcl-xL protein levels in the
mitochondria of small preantral follicles, an increase in Bcl-xL protein levels in the
cytosol of small preantral follicles, an increase in the ratio of Bax to Bcl-xL protein levels in mitochondria,
a decrease in the ratio of Bax to Bcl-xL protein levels in cytosol, and a shift of cytochrome c
from the mitochondria of small preantral follicles to the cytosol. All of these effects occurred
specifically in VCD-targeted small preantral follicles, and only occurred after repeated
administration of VCD. Thus, the anti-apoptotic Bcl-xL proteins would be blocked by an
increase in non-phosphorylated Bad protein. The increase in Bax to Bcl-xL protein ratio in the
mitochondria with an increase in cytoplasmic Bcl-xL suggests a decrease in Bax that may be
explained by the loss of Bax-producing follicles through apoptosis. The increase in cytochrome
c in the cytosol is consistent with accelerated apoptosis.[30]
Mitogen-activated protein kinases (MAPK) include extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 kinase. These MAPKs are regulated by signal transduction pathways that control different processes, including apoptosis. While ERK is involved in cell proliferation, the JNK and p38 kinase are activated by pathways that induce apoptosis. Activator protein-1 (AP-1) is a transcription factor that is phosphorylated by MAPKs and reacts with AP-1 sites on DNA sequences. Once activated AP-1 may be involved in apoptosis, but it reacts differently in different types of cells. The nuclear AP-1 DNA binding complex present in rat ovarian follicles contains c-Jun and phospho-c-Jun proteins. The c-Jun protein is a member of the AP-1 family and is a transcription factor that enhances the transcriptional activation of nuclear AP-1 when it is phosphorylated. When rats are administered a single dose of VCD (80 mg/kg, ip) there is a decrease in JNK activity in small preantral follicles. When female Fischer rats (28 days old) are administered VCD (80 mg/kg, ip) for 10 or 15 days JNK and p38 kinase activities are increased in small preantral follicles. These protein levels are greater after 10 days of VCD than after 15 days of VCD. This is consistent with the fact that by day 10 of VCD administration small preantral follicles are beginning to undergo apoptosis, but by day 15 most preantral follicles have been destroyed. After 15 days of VCD administration, rat small preantral follicles exhibit an increase in phospho-c-Jun protein. AP-1 binding activity is increased in small preantral follicles after one dose of VCD, decreased after 10 days of VCD, and decreased after 15 days of VCD (but to a lesser extent than after 10 days). This means that VCD alters transcription factors that regulate AP-1 binding and activity and therefore how signals are relayed to the nucleus. This also means that increased phospho-c-Jun is associated with a reduction of the c-Jun transcriptional regulating capacity and therefore a
decreased AP-1 DNA binding activity in rat small preantral follicles. These transcription factors are therefore characteristic of apoptosis in rat small preantral ovarian follicles.\cite{31}

Acid sphingomyelinase (ASMase) is an enzyme that generates ceramide (an oxidative stress sensor) and is required for oocyte death under normal conditions and after chemotherapeutic drug exposure. ASMase knockout mice (27 days old) exhibited the same primordial and primary follicle loss when treated with VCD (80 mg/kg, ip) as controls. Although this eliminates ASMase as a mediator of VCD-induced ovarian follicle loss, it does not eliminate ceramide because it can be generated by other sources. The transcription factor aromatic hydrocarbon receptor (Ahr) is required for PAH (environmental contaminants) to destroy primordial and primary mouse ovarian follicles. Mice that do not produce Ahr exhibit better oocyte survival during development. Ahr knockout mice (35 days old) do exhibit the same primordial and primary follicle loss after VCD exposure as mice. The proapoptotic Bax protein has been identified as a modulator of follicle loss due to VCD administration. Bax knockout mice (27 days old) have significantly decreased primordial and primary follicle loss compared to controls. Caspase-2 is a protease necessary for oocyte destruction during development and after anticancer drug exposure. Caspase-2 knockout mice (27 days old) exhibit significantly decreased primary follicle loss due to VCD administration. Caspase-3 is a protease that is involved in oocyte and granulosa apoptosis due to VCD exposure. Caspase-3 knockout mice (27 days old) exhibit significantly decreased primary follicle loss after VCD administration as compared to controls. Taken together, this data indicates that caspase-2 and caspase-3 are activated by VCD as part of primary follicle destruction, and Bax is involved in both primordial and primary follicle loss due to VCD. However, other pathways must be involved in VCD-
induced follicle loss because the Bax, caspase-2, and caspase-3 knockout mice only exhibited reduced levels of follicle loss and were not completely protected from oocyte destruction.\textsuperscript{[32]}

Mouse pups that overexpress \textit{bcl-2} exhibit 50\% more primordial follicles than wildtype pups. Pregnant mice that overexpress \textit{bcl-2} were dosed with VCH (500 mg/kg, gavage) for 11 days, on days 8-18 of pregnancy. They exhibited no change in follicle counts, but their pups (8 days old) exhibited a decrease in primordial, primary, and secondary follicles as compared to control \textit{bcl-2} overexpressing pups. When VCH is administered to wildtype adult mice (500 mg/kg, gavage) for 11 days on days 8-18 of pregnancy, their pups (8 days old) exhibit reduced secondary follicle counts. VCH administered to non-pregnant wildtype adult mice (age not given) resulted in a loss of secondary follicles. Non-pregnant adult \textit{bcl-2} overexpressing mice (age not given) administered VCH in the same manner developed primordial, primary, and secondary follicle loss. Apoptosis, as detected by TUNEL staining was increased in the \textit{bcl-2} overexpressing control pups and the pups whose mothers were administered VCH. This research indicates that \textit{bcl-2} overexpression results in the loss of small preantral follicles in neonatal mice exposed to VCH in utero and in non-pregnant adult mice. It is possible that \textit{bcl-2} overexpression does not prevent apoptosis of neonatal primordial follicles, but rather results in high numbers of unhealthy follicles that are highly susceptible to VCH-induced apoptosis. These results also indicate that fetal oocytes are more susceptible to VCH damage than adult follicles. It is assumed that longer exposure to VCD would have resulted in follicle loss in wildtype mice.\textsuperscript{[33]}

Growth and differentiation factor 9 (GDF9) and bone morphogenetic factor 4 (BMP4) are known to promote rat ovarian follicle survival. Kit Ligand (KITL) is another growth factor that signals the oocyte through the KIT receptor to stimulate oocyte growth and prevent apoptosis in primordial follicle oocytes. It was determined that 20 \textmu M of VCD administered to Fischer 344
rat (28 days old) ovaries \textit{in vitro} mimic a time point when follicle loss is impending but has not yet begun \textit{in vivo} for rats treated with VCD for 12 days (80 mg/kg, ip). By oligoarray analysis, 33 genes were different between control small preantral follicles and VCD treated small preantral follicles. There were 25 genes that were different between control cultured whole ovaries and whole ovaries cultured with VCD. For both the \textit{in vivo} and \textit{in vitro} ovarian systems, VCD exposure altered 14 genes. The mRNA expression of 7 genes was different between control and VCD treated large secondary and antral follicles. None of the altered genes are associated with follicular signaling pathways. Both \textit{in vivo} and \textit{in vitro} there were decreases in the mRNA expression of BMP4 receptor and KITL receptor. Ovaries cultured in VCD had less follicle loss with addition of KITL, especially with regard to primary follicles. Cultured rat ovaries demonstrated greater primordial follicle loss when exogenous KITL was added (400 ng/mL) along with VCD (30 μM), but decreased primary follicle loss. Ovaries treated with KITL displayed transient follicles with enlarged oocytes and flattened granulosa cells that were recruited from primary follicles. Thus it appears that KITL acts to protect primary follicles from VCD and that VCD accelerates primordial oocyte growth. When ovaries were cultured with VCD (30 μM), there was a decrease in KIT mRNA beginning on day 4 of culture, followed by an increase in KITL mRNA beginning on day 6 of culture. KIT ovarian protein was decreased with addition of VCD but KITL protein was increased after 8 days of VCD exposure. Staining for KIT was found to be localized inside oocytes and greatest in primordial and primary follicles. Ovaries incubated with VCD exhibited decreased KIT staining intensity. Thus, VCD appears to interact with the KIT/KITL pathway that is protective of primary follicles. VCD administration causes a decrease in KIT and an increase in KITL expression. GDF9 and BMP4 signaling pathways do not appear to be involved in VCD ovotoxicity.\cite{34}
It is known that 17β-Estradiol (17β-E2) is involved in apoptosis in many different types of tissues. E2 is the endogenous ER ligand. Since E2 levels normally increase as ovarian atresia decreases, it is suspected that E2 is normally involved in controlling apoptosis. Genistein is an ER agonist, while 4-hydroxytamoxifen is an ER antagonist. When female Fischer 344 rats (28 days old) are dosed with VCD (80 mg/kg, ip) for 15 days along with E2, primary follicle counts are equal to those of control rats. With administration of E2 alone, primary follicle counts are increased compared to controls. Genistein administration produces results similar to those for E2. With VCD, E2, and 4-hydroxytamoxifen administration primary follicle loss is not prevented. VCD administration alone results in increased caspase-3 activity, but treatment with genistein (either with VCD or alone) results in caspase-3 activity levels that are similar to control levels. Small preantral follicles exhibited ERβ protein in greater amounts than ERα protein. ERα protein is localized in the nucleus of oocytes, while ERβ protein is evident in the cytoplasm and nuclei of oocytes and granulosa cells of primordial and small primary follicles. In larger follicles ERβ protein is only apparent in the nucleus. VCD does not alter ER binding of E2, ER distribution, or ER number. This implies that the mechanism by which 17β-E2 protects primary follicles does not involve a direct interaction between 17β-E2 and VCD. Therefore, E2 likely prevents ovotoxicity induced by VCD through a receptor-mediated pathway. The E2 agonist genistein prevents the follicle loss and increased caspase-3 activity induced by VCD, suggesting that E2 prevents ovotoxicity through reduced apoptosis. Since 17β-E2 does not appear to protect primordial follicles it is possible that VCD does not directly kill primordial follicles but rather promotes their development into larger follicles.[35]

The aryl hydrocarbon receptor (AhR) is a transcription factor that regulates cell cycle, apoptosis, and oxidative stress. The AhR is expressed in the ovary and plays a role in follicular
atresia. Female Fischer 344 rats (28 days old) dosed with VCD (80 mg/kg, ip) and the AhR antagonist alpha-naphthoflavone (ANF) had the same numbers of primordial and primary follicles as control rats. In mice, ANF had no effect on follicle loss. VCD administration increased AhR mRNA in small preantral follicles for rats but not mice. AhR protein is found in the nucleus of granulosa cells and oocytes of rats and mice. VCD administration induces an increase in AhR protein in the nuclei of rat primordial and primary oocytes. It is therefore likely that AhR is involved in rat but not mouse VCD-induced ovarian toxicity.\textsuperscript{[36]}

Nuclear factor erythroid 2-related factor 2 (Nrf 2) is a leucine zipper transcription factor that is involved in controlling the expression of protective genes, like antioxidants. Nrf 2 null mice (28 days old) treated with VCD (0.57 mmol/kg/d, ip, 15 d) exhibit greater follicle loss than wildtype mice administered VCD. Murine hepatoma cells incubated with VCD (0.5, 1, 2 mM) for 18 hours exhibit a dose dependent increase in apoptosis. In vivo, Nrf2 null mice exhibit apoptosis, mostly in large growing follicles, at increased numbers compared to wildtype mice. The litter size of Nrf2 mice is dramatically reduced in Nrf2 null mice and decreased by approximately 25% in wildtype mice. LH levels are increased by VCD administration in wildtype mice, while LH and FSH levels are increased in Nrf2 null mice. Nrf2 is found to be a major regulator of mEH expression, so that Nrf2 null mice would display a reduced detoxification capability and therefore an increased susceptibility to the effects of VCD. VCD administration increases oxidative stress, with Nrf2 null mice exhibiting greater oxidative stress than wildtype mice. Reactive oxygen species are produced as a result of a synergistic effect between VCD and the lack of Nrf2. VCD also increases the expression of oxidative stress marker genes in ovaries and hepatoma cells. However, without Nrf2 this induction in the ovaries does not occur, suggesting that Nrf2 is necessary for VCD induced oxidative stress and
transcription of oxidative stress marker genes in the ovaries. VCD administration increases the amount of Nrf2 protein in mice, implying that VCD stabilizes Nrf2 just like any other oxidative stressor. Therefore, VCD causes oxidative stress that induces an Nrf2 response in the ovaries as a protective measure against reactive oxygen species. Foxo3a is a forkhead transcription factor that is involved in suppressing the early stages of follicular growth. VCD treatment increases Foxo3a expression in wildtype and, even more so, in Nrf2 null mice. In hepatoma cells and wildtype ovaries, VCD induces Foxo3a mRNA expression. Since the ovaries of Nrf2 null mice do not exhibit increased expression of Foxo3a with VCD administration, it is implied that Foxo3a regulation is independent of Nrf2 in the presence of VCD. This occurs because VCD inhibits the proteasomal degradation of Foxo3a protein. This would suggest that Foxo3a induction in the presence of VCD is a response to increased oxidative stress.[71]

In vitro Effects of VCD on the Ovary

Preantral follicles, interstitial cells, hepatocytes, and adrenal cells from immature female Fischer rats (21-28 days old) were incubated with VCD at concentrations of 38, 76, and 152 μM for 2, 4, 6, and 24 hours. The viability of the interstitial cells, hepatocytes, and adrenal cells was never compromised. The viability of the small preantral follicles (75-100 μm) was reduced at VCD concentrations of 76 and 152 μM after 4 hours. The viability of the granulosa cells of the small preantral follicles was reduced when the follicles were incubated with 38 μM at 6 hours. The viability of the granulosa cells of the large preantral follicles (100-250 μm) was reduced at 24 hours with all three concentrations of VCD. The tetrol metabolite of VCD never affected the viability of the preantral follicles. Large preantral follicles were able to convert VCD to the tetrol metabolite at amounts 1.5-fold greater than the small preantral follicles, 4-fold greater than
interstitial or adrenal cells, and the same as hepatocytes. Preantral follicles, interstitial cells, hepatocytes, and adrenal cells from mature rats (51-58 days old) were incubated with VCD at a concentration of 76 μM for 1 hour. The small and large preantral follicles converted VCD to similar amounts of the tetrol metabolite. Interstitial cells converted VCD to the least amount of the tetrol metabolite. The adrenal cells converted VCD to 1.6 times the tetrol metabolite as interstitial cells, while the hepatocytes converted VCD to 10 times the amount of the tetrol. Hepatocytes produced the greatest amount of tetrol from VCD. Primary follicles (25-50 μm) conversion of VCD to tetrol was 8 times less than large and small preantral follicle conversion of VCD to tetrol. Small growing follicles with two to three layers of granulosa cells (50-75 μm) converted VCD to amounts of tetrol that were three times less than the amounts converted by the small and large preantral follicles and three times greater than the amounts converted by primary follicles. The adult rat preantral follicles, interstitial cells, hepatocytes, and adrenal cells converted VCD to greater amounts of tetrol than the same cell types in the immature rats. This means that the smallest follicles are not able to detoxify VCD to the tetrol metabolite as well as larger follicles. This also means that immature rats are less capable of detoxifying VCD than adult rats. Since granulosa cells are directly compromised by VCD and interstitial cells have a small capacity to detoxify VCD, it would seem that that the small preantral follicles that are surrounded by these cells would be particularly susceptible to VCD-induced ovotoxicity.\[^{44}\]
Fig. 1.15  Micrographs depicting the changes in primordial follicle counts observed with *in vitro* VCD treatment. The ovaries of postnatal day 4 Fisher 344 rats cultured in the absence of VCD (A) display greater numbers of primordial and small primary follicles (indicated by the white arrows) as compared to ovaries cultured in 30 μM VCD (B).[63]

Incubation of Fischer 344 rat ovaries (4 days old) in VCD (1, 3, 10, 30, 100 μM; 2, 4, 8, 10, 15d) did not result in necrosis or grossly altered morphology. It did result in a concentration-dependent loss of primordial and small primary ovarian follicles. This occurred with VCD concentrations as low as 30μM as early as 8 days of culture. LDH assays indicate that the cultured ovaries were not undergoing necrosis. This data supports the idea that VCD has direct effects of ovarian follicles, without extraovarian metabolism or hormonal influences. Staining for PCNA, a marker of actively proliferating granulosa cells, indicates that ovaries incubated in VCD do not differ from controls in this regard. PNCA staining appeared in the nuclei of
granulosa cells in growing follicles of ovaries incubated with VCD or control medium. Impending cell death was detected using immunohistochemistry markers for TUNEL, a marker for DNA degradation, and active caspase-3, a marker for apoptosis. In ovaries incubated in 30 μM VCD there were significantly more cells that stained positive for TUNEL and active caspase-3. This staining occurred primarily in granulosa cells of primary or secondary follicles. Positive staining for TUNEL in eosinophilic degenerating follicles indicate that DNA degradation is also occurring in the oocytes of follicles.[62]

Ovaries from 4 day old Fischer 344 rats can remain viable during co-culture with VCD (10, 20, or 30 μM) for 16 days. Ovarian follicles progress through the developmental pathway until reaching the small secondary stage. A concentration-dependent decrease in the numbers of primordial and small primary follicles occurs in rat ovaries co-cultured with VCD (10, 20, or 30 μM) for 15 days. During the first 24 or 48 hours of co-culture with VCD, the rat ovaries have significantly reduced numbers of primordial and small primary follicles as compared to controls. This is true regardless of whether the VCD is freshly added to the culture or is allowed to pre-incubate in culture wells. This indicates that VCD does not spontaneously degrade. When ovaries were co-cultured with VCD (30 μM) that had been pre-incubated with different ovaries, the decrease in follicle counts was less but not significantly different than controls. This indicates that the ovary is able to metabolize VCD. When antioxidants are added to the culture, there is no change in VCD-induced follicle depletion which suggests that the mechanism of VCD-induced ovotoxicity does not involve oxidative stress. This study also indicates that there is a threshold concentration of VCD above which the ovary does not have the ability to metabolize VCD[63]
The small preantral follicles (25-100 μm) of immature (21-28 days old) Fischer 344 rats exhibit a significant decrease in viability when incubated in VCD (38, 76, 152 μM) for 6 hours. At the 152 μM VCD concentration, the viability of small preantral follicles is compromised by 4 hours of incubation. The effects of VCD on protein synthesis are measured by the rate of ³H-leucine incorporation into proteins. Small preantral follicles exhibit a decreased rate of protein synthesis to 78.8±3.9% and 52.6±9.3% of control values at VCD concentrations of 76 and 152 μM, respectively. The small preantral follicles isolated from rats treated with VCD (80 mg/kg, ip, 10 d) exhibit decreased viability after 6 hours of incubation in VCD (76 μM). This decrease in viability is greater than that expressed in small preantral follicles isolated from untreated rats. Small preantral follicles incubated in VCD (76 μM) exhibit decreased protein synthesis rates for follicles isolated from untreated and VCD (80 mg/kg, ip, 10 d) pre-treated rats. For untreated rats the rates of protein synthesis in isolated small preantral follicles was reduced at 3 hours but stimulated at 6 and 10 hours of incubation with VCD, while the follicles from pre-treated rats exhibited protein synthesis inhibition beginning at 3 and extending to 10 hours of incubation. This suggests that the effects of VCD on untreated small preantral follicles are reversible, while the effect of VCD incubation of pre-treated small preantral follicles is irreversible. The growing preantral follicles (100-250 μm) of untreated rats exhibited significantly increased protein synthesis rates with 3 hours of incubation in VCD (76 μM). Adrenal cells, isolated granulosa cells, isolated oocytes, and liver cells of untreated rats were unaffected by 3 hour incubation in VCD. Ovarian interstitial cells and small preantral follicles of untreated rats displayed decreased protein synthesis rates with 3 hours of VCD incubation. This data suggests that daily dosing with VCD results in a greater effect of VCD on small preantral follicles in vitro. Also, the effect of VCD on small preantral follicles in vitro requires granulosa cells and oocytes to remain
associated. This also suggests that granulosa cells are involved in the ovotoxicity resulting from VCD exposure.[69]

**Fertility in VCD-treated Rodents**

Perimenopause is the gradual onset of ovarian failure that occurs during the four to ten year period before menopause, when the ovary becomes completely devoid of follicles. Perimenopause affects conception and pregnancy, resulting in increased incidences of miscarriages, ectopic pregnancies, fetal abnormalities, chromosomal abnormalities, and twin births. Older women can also exhibit subfertility wherein they have a lower ability to reproduce and there is a longer period of time required to become impregnated. This is because the pool of primordial follicles is becoming depleted with time and the remaining follicles are of lower quality. It was found that female C57BL/6J mice (28 days old) dosed with VCD (160 mg/kg/d, ip) for 17 days exhibited ovarian failure (at least 15 consecutive days of metestrus/diestrus or diestrus) between 36 and 56 days after VCD dosing. This time period was considered perimenopause. VCD treated animals initially exhibited lower body weights than control mice, but eventually exceeded the body weights of control animals 180 days post treatment. VCD treated animals exhibited lower uterine and adrenal gland weights than control animals. This was determined to be a result of ovarian failure and not a direct result of the VCD. In mice mated with males immediately after VCD administration, 5 became pregnant after the first mating, 5 became pregnant after the second mating, and 4 exhibited copulatory plugs but did not become pregnant. These VCD mice were determined to be analogous to women in the early stages of perimenopause, and exhibited subfertility. All control animals became pregnant. Those VCD mice that became pregnant had smaller percentages of viable fetuses than control
mice. Although the VCD and control mice had similar conception rates, the VCD treated mice displayed more resorbed fetuses than the control mice. The four VCD mice that did not become pregnant are said to be representative of interindividual variation. In mice mated with males 20 days post VCD administration, all mice were cycling and exhibited copulatory plugs. Only 1 out of the 8 VCD treated animals became pregnant, while 4 out of the 6 control mice became pregnant. The one VCD mouse that became pregnant did not develop any live fetuses and 5 resorbed fetuses. Compared to control mice with evidence of all types of follicles, VCD treated mice had no follicles. Those VCD mice that became pregnant showed only an occasional antral follicle. Those VCD-treated mice that did not become pregnant had no corpus luteum, which form at sites of ovulation. These VCD-treated mice are said to be analogous to women in the later stages of perimenopause, and exhibited impaired fertility and infertility despite intact ovarian function.[48]

When B6C3F1 male mice (28 days old) are administered VCD (320 mg/kg/d, ip) for 5, 10, 20, 25, or 30 days to determine a time response to VCD, it was found that the mice exhibited testicular damage in the form of decreased testis weights, histologic lesions, loss of spermatogonia and spermatocytes, and great loss of germ cells, in a time dependent manner. Sixty days post-VCD testis weights returned to 60% of control weights, while the seminiferous tubules were completely repopulated by germ cells 30 days after VCD administration. When male mice were administered VCD for 30 days at concentrations of 40, 80, 160, or 320 mg/kg/d (ip), it was found that there was a dose-dependent decrease in the testis weight. The weight of the seminal vesicles was also decreased at a VCD dose of 320 mg/kg/d. Mice treated with 160 mg/kg/d of VCD exhibited significantly or completely reduced numbers of spermatozoa in the epididymal tubules. Since the germ cells affected by VCD administration were those that are
involved in DNA synthesis and cell replication (differentiated spermatogonia and preleptotene spermatocytes), it is hypothesized that VCD disrupts the germ cells through DNA binding and interference with the process of DNA synthesis. Since spermatogonial stem cells were not affected by VCD, these cells were able to repopulate the seminiferous tubules. VCH and the VCH-monoepoxides had no effect on male mice. In addition, male rats did not exhibit any effects as a result of VCD administration.[49]

Female Simonson albino rats (28-45 days old) administered VCD (20-40 mg/kg estimate, water consumption) exhibit no weight changes relative to controls. The fertilizability of the oocytes was not affected and the number of ovulating females remained unchanged. The oocytes of the females exposed to VCD were slightly more fragile than controls, as evidenced by the numbers of remaining oocytes after removal of the zona pellucida.[57]

**Assessing Ovarian Sections of VCD-treated Mice for Follicle Counts**

To analyze if random counting of ovarian follicles produces reliable estimates of follicle counts, the ovaries of C57BL/6N and B6C3F₁ mice (7-10 weeks old and 4-6 weeks old, respectfully) treated with VCD were sectioned. These sections were first counted serially by counting every tenth section for a total of 30 to 60 sections per ovary. From these 30 to 60 sections, 5 sections were chosen to be randomly counted by a random number generator. These random section counts were then compared to the serial counts. Follicles were classified as either primordial, growing, or antral. The random and serial sections produced the most similar results for primordial follicles; the random counts were within 10% of the serial counts. It can be concluded that counting of 5 randomly selected ovarian sections is sufficient enough to estimate primordial follicle numbers. Counting 5 random serial sections appears sufficient to yield
qualitative estimates of growing follicle numbers in mice administered ovotoxicants, but does not appear sufficient to detect the loss of antral follicles.[51]

**Utility of VCD-induced Ovarian Failure Model**

VCD has been used to try and develop a nonhuman primate model of the menopausal transition. VCD (80 mg/kg, 160 mg/kg, 250 mg/kg, im) was administered to cynomolgus macaques (8-12 years old) for 15 days. These primates exhibit many more similarities to human females because they undergo menstrual cycles and not estrus cycles like rodents. This potentially makes them a more ideal model for the study of human disease. In the high dose VCD group, swelling and inflammation at the injection site were observed with the development of persistent scar tissue. The primates in this group also exhibited a transient increase in the liver enzymes aspartate amino serum transferase (AST) and amino alanine transferase (ALT).

Primates in the low dose VCD group did not exhibit significantly different ovarian follicle counts immediately after VCD dosing. The 160 mg/kg/d VCD group exhibited an approximately 50% decrease in the number of primordial and primary follicles. The high dose VCD group exhibited almost a complete loss of all follicle types, immediately following the 15 day administration period. At necropsy, two out of four monkeys exhibited granulomatous lesions (semimembranosis and semitendinosis muscle) at the site of VCD injection.[45]

However, in subsequent attempts to replicate this work, the researchers have failed to induce primary and primordial follicle loss through intramuscular injection of VCD in cynomolgus macaques. They have also found that the intramuscular injection of VCD results in severe irritation in the tissue at the site of injection.[46]
VCD has also been used as a model of perimenopause in mice during studies of atherosclerotic lesions. It was found that female C57BL/6J LDLR -/- (low-density lipoprotein receptor-deficient) mice (28-35 days old) administered VCD and 17β-estradiol (E2) had smaller innominate atherosclerotic lesions than ovariectomized (OVX) C57BL/6J LDLR -/- mice administered 17β-estradiol. These mice had similar aortic atherosclerotic lesions. VCD did not induce any changes in plasma total cholesterol levels, HDL cholesterol levels, or triglyceride levels. FSH and LH levels are higher in VCD treated animals (and menopausal animals) because the estrogen once produced by the follicles is gone and cannot provide negative feedback to the pituitary. Elevated LH levels stimulate cells in the residual tissue of the follicle-deplete ovary to continue producing steroids such as progesterone and androgen. The OVX and VCD mice in this study both exhibited similar elevated levels of FSH as compared to cycling control mice. Administration of exogenous E2 only slightly reduced these levels. Cycling controls, OVX, and VCD animals exhibited no detectable levels of E2. Plasma estradiol levels were within the physiological limits expected for cycling mice in VCD mice administered E2, but were increased in OVX mice given E2. This may imply that the residual interstitial cells of the follicle deplete ovary may metabolize estradiol. Androstenedione levels were similar between vehicle and VCD mice. Mice administered VCD and E2 exhibited lower androstenedione levels than VCD mice. OVX mice exhibited lower androstenedione levels than control while OVX mice given E2 exhibited the highest androstenedione levels. This implies that E2 blocks the residual interstitial cells of ovaries in VCD treated mice from producing androstenedione. This model therefore may be more reflective of the endocrine profile for perimenopausal women, in which androgen and progesterone production from residual ovarian tissue occurs. This shows that the residual
tissue of the VCD follicle deplete ovary acts to influence exogenous estradiol and, in this case, ameliorate the progression of lesions in the innominate artery.\textsuperscript{[47]}

The VCD model has also been used to study the effects of diabetes during perimenopause. It was found that peri-ovarian failure female B6C3F\textsubscript{1} mice (28 days old) exhibit a significant increase in blood glucose compared to control mice, when injected with steptozotocin (STZ) to induce type I diabetes. These blood glucose levels displayed a trend to be higher, but were not significantly higher than regularly cycling diabetic mice. The glucose levels of the peri-ovarian failure mice were significantly lower than those of the post-ovarian failure mice. There are also changes in gene expression during the peri-ovarian failure period, as compared to cycling diabetic mice.\textsuperscript{[61]}
BONE

Bone Turnover

Bone is made up of specialized cells, mineralized connective tissue matrix, non-mineralized connective tissue matrix, the bone marrow cavity, vascular canals, canaliculi, and lacunae. Bone is comprised of organic and inorganic components. Over 90% of bone is type I collagen, while the rest is comprised of noncollagenous proteins that contain the mineral substituted hydroxylapatite. Bone tissue is constantly being remodeled through a cycle of bone resorption and bone formation. Specialized cells are involved in these processes; osteoclasts are involved in bone resorption and osteoblasts are involved in bone formation. The two processes of bone resorption and formation are coupled together so that under normal circumstances a balance is achieved between the amount of bone lost and the amount of bone formed. This balance is regulated using hormones, such as parathyroid hormone (PTH) and vitamin D, and local mediators, such as cytokines and growth factors. The resorption phase of bone remodeling in humans occurs over a period of approximately ten days, while the formation phase of bone remodeling occurs over a period of up to three months. This can be compared to the process of bone turnover in rats, which lasts approximately 40 days.

Fig. 1.16 The bone remodeling cycle.
Remodeling can be defined in terms of an initiation phase, a transition phase, and termination. In the initiation phase osteoclast precursors are recruited, osteoclasts differentiate, and bone is resorbed. In the transition phase, bone resorption is inhibited as osteoclasts undergo apoptosis. During this time, osteoblasts are recruited, differentiate, and bone is prepared for osteoid formation. In the termination phase, osteoid is deposited and the bone matrix is mineralized.[93]

Fig. 1.17 Depiction of the three-phase model of bone remodeling. The three-phase model of bone remodeling consists of periods of initiation, transition, and termination. During initiation, osteoblasts induce the formation of osteoclasts through RANK-L interaction. These osteoclasts initiate bone resorption. During transition, coupling factors, diffusible factors, membrane bound molecules, and bone matrix factors initiate the switch from bone resorption to bone formation. During termination, osteoblasts fill in the resorbed lacuna with bone.[93]

When bone is growing during the developmental process its shape is modeled as bone is removed at certain sites and deposited at different sites. After the shape of bone is established, bone is remodeled as bone is removed and deposited at the same site. The adult skeleton is completely remodeled every ten years. It is hypothesized that the purpose of this remodeling is
to repair accumulated damages and preclude the aging process. Osteoclasts and osteoblasts that work together in the act of remodeling temporarily form a collective structure termed the basic multicellular unit (BMU). The BMU is approximately 1-2 mm in length and 0.2-0.4 mm in width. It is composed of osteoclasts in the front, osteoblasts in the back, a central vascular capillary, a nerve supply, and connective tissue. Approximately 1 million BMUs are functioning at any given moment, with a total of 3-4 million BMUs being formed each year. The BMU is formed at a point of origination and advances toward a target area of bone in need of remodeling. In cortical bone the BMU will resorb and replace a tunnel of bone. In cancellous bone the BMU will resorb and replace a trench of bone on the trabecular surface. Osteoclasts acidify and then proteolytically digest bone. As the BMU progresses the osteoblasts will move in place over the area of resorbed bone. The osteoblasts will secrete osteoid that will eventually be mineralized into new bone. BMUs survive for 6-9 months, with new osteoclasts and osteoblasts being continuously supplied from bone marrow progenitors. In younger individuals, the amount of bone restored is equal to the amount of bone lost. In older individuals, the amount of bone formed by osteoblasts may be less than the amount of bone resorbed by osteoclasts.

The actions of osteoblasts and osteoclasts are coupled in the BMU and bone formation will begin as resorption advances. Two models have been developed to explain this. In the serial model the resorbed bone or the strain induced on bone by the process of resorption stimulate osteoblast precursors to proliferate and differentiate. In the parallel model, osteoclasts and osteoblasts proliferate and differentiate at the same time due to an external signal.
Fig. 1.18 Depiction of the serial and parallel models of osteblast and osteoclast development. GF stands for growth factors that are released from the matrix of resorbed bone, PreOC stands for preosteoclast, PreOB stands for osteoblast progenitors.[90]

**Cortical versus Cancellous/Trabecular Bone**

Cortical, or compact, bone is relatively solid with few spaces. The spaces that do exist house canaliculi ("channels containing cell processes that connect the cells with each other and with the nearest blood channel"), osteocytes, blood vessels, and erosion cavities. In contrast, cancellous bone contains large spaces. Therefore cancellous bone contains a larger percentage of its total volume that is not occupied by bone tissue. Rather, this area is filled with marrow. Cancellous bone is comprised of lamellar bone with collagen fibrils and minerals arranged in sheets. At the surface of a bone, cancellous bone is usually arranged as many sheets. Deep within a bone, cancellous bone is usually arranged randomly with many cylindrical trabecular struts connecting the sheets. Each trabecular strut is approximately 1 mm in length. These cylindrical struts usually extend for approximately 0.1 mm before they connect with another strut. This joining of trabecular struts creates a smooth, continuous curve between one trabecular strut and the next. Trabecular diameter is directly proportional to body mass which
is directly proportional to trabecular length. Cancellous/trabecular bone is always weaker and less stiff than cortical bone. It is primarily found at the ends of long bones, in the medullary cavity of some long bones, as a filling in short and flattened bones, and under protuberances where tendons attach. Trabecular bone is metabolized differently than cortical bone. The femoral proximal and distal metaphyses, and the tibial proximal metaphysis contain high percentages of trabecular bone. The femoral and tibial diaphyses contain mostly cortical bone.

**Osteoclasts**

Mature osteoclasts are multinucleated cells that are approximately 50-100 mm in diameter. They are formed through the fusion of mononuclear progenitors derived from the hematopoietic cells of the monocyte/macrophage family. Osteoclast precursors reach bone through the circulatory system. Chemokines attract osteoclast precursors to bone. One example is monocyte chemoattractant protein-1 (MCP-1) that is secreted by osteoblasts. MCP-1 is secreted in response to pro-inflammatory cytokines like TNF-α and IL-1b, and parathyroid hormone (PTH). RANK-L induces the expression of MCP-1 receptors on osteoclast precursors.

Osteoclasts have a ruffled border that mediates the resorption of the calcified bone matrix. The ruffled border is surrounded by a clear zone with cytoplasm that contains bundles of actin-like filaments. An ATP-driven proton pump (vacuolar H1-ATPase) in the ruffled border membrane creates an acidic microenvironment at the site of bone resorption. The osteoclast then endocytoses the degraded bone matrix and eventually releases the degraded components in an area opposite the site of resorption. Osteoclasts have high levels of tartrate-resistant acid phosphatase (TRAP), which is a phosphohydrolase enzyme. Therefore TRAP can be used as
TRAP-5b is the TRAP isoform characteristic of osteoclasts. Osteoclasts can be stained for TRAP as a means of assessing osteoclast activity. The function of TRAP in osteoclasts is unknown. However, TRAP is necessary for the processes of bone resorption and collagen turnover. Under normal conditions, TRAP coats resorption lacunae. Mice that overexpress TRAP display increased rates of bone formation, suggesting that TRAP stimulates this process. This would allow osteoblasts to commence bone formation in lacunae where TRAP is present but there are no osteoclasts.

Osteoclasts have a lifespan of approximately 2 weeks, and die by apoptosis. Osteoclasts undergo apoptosis when bone formation commences, through a Bim/caspase-3 or an estrogen-induced Fas ligand dependent pathway. The high calcium levels that result from resorption induce osteoclast apoptosis.

Marrow stromal cells or their osteoblast progeny must be present in order for osteoclasts to form. These two accessory cells allow macrophages to become osteoclasts because they secrete two stimulatory molecules: macrophage colony-stimulating factor (M-CSF) and receptor for activation of nuclear factor kappa B (NF-κB) (RANK) ligand (RANK-L). Early osteoclast precursors express a receptor (c-Fms) for M-CSF. When M-CSF binds it signals the osteoclast precursor to survive and proliferate. Once M-CSF has signaled the osteoclast precursors to proliferate, RANK-L will stimulate these cells to commit to the osteoclast phenotype. RANK-L has a high affinity for RANK. Hematopoietic osteoclast progenitors express RANK, while preosteoblastic cells, stromal cells, and T lymphocytes express RANK-L on their surface. 1,25-(OH)2D3, PTH, PTHrP, gp130 activating cytokines (e.g., IL-6, IL-11), and IL-1 can all induce stromal/osteoblastic cells to express RANK-L. Thus, osteoclastogenesis requires that the osteoclast precursors come in contact with stromal cells or osteoblasts.
Osteoblasts and osteoclasts can interact through ligands and receptors in their membranes that interact to produce intracellular signals. They can also form gap junctions to allow molecules to pass between them. Growth factors, cytokines, chemokines, and other paracrine factors can be secreted by osteoblasts and osteoclasts to affect one another. Osteoblasts and osteoclasts can also interact through the release of growth factors from the bone matrix during the process of bone resorption by osteoclasts. These growth factors include TGF-β, bone morphogenetic proteins (BMPs), and insulin-like growth factor (IGF)-II.\cite{93}

Osteoprotegerin (OPG) is a glycoprotein that inhibits osteoclastogenesis because it acts as a competitive inhibitor of RANK-L.\cite{90} How much bone is reabsorbed depends on the balance between RANK-L and OPG. In vitro, macrophages can be stimulated into osteoclasts when exposed to M-CSF and RANK-L. Tumor necrosis factor (TNF) stimulates osteoblasts to express M-CSF and RANK-L in vivo. Interleukin-1 (IL-1) stimulates marrow stromal cells to express M-CSF; this stimulation is inhibited by estrogen. Therefore, during the post-menopausal period loss of estrogen will result in an increase in M-CSF resulting in an increase in osteoclastogenesis.\cite{89}

Osteoclast precursors display immunoglobulin-like receptors, such as OSCAR, that co-stimulate the RANK signaling pathway. The RANK signaling pathway induces a transcription factor cascade in osteoclasts that includes NF-κB, AP-1 (c-Fos), and NFATc1. PTH, parathyroid hormone-related peptide (PTHrP), TNF-α, IL-1, IL-11, thyroid hormone, 1,25-(OH)₂ vitamin D₃, and prostaglandin E₂ are all factors that can enhance osteoclastogenesis through induction of RANK-L in osteoblasts. In contrast, OPG binds to RANK and inhibits the RANK/RANK-L signaling pathway.\cite{93} Prostaglandins are involved in the production of cyclooxygenase (COX)-1 and COX-2. The inducible COX-2 form is associated with RANK-L expression. COX-2 also

prevents the expression of granulocyte macrophage-colony stimulating factor (GM-CSF), which inhibits the differentiation of osteoclast precursors. Therefore pro-inflammatory substances like IL-1, TNF-α, and prostaglandins (PGs) increase the process of bone resorption.\[91\]

Fig. 1.19 Depiction of osteoclast maturation. Osteoclast precursors differentiate into mononuclear osteoclasts. These cells will fuse to form a multinucleated osteoclast. All steps of the osteoclast maturation process require the presence of RANK-L, which is secreted by the osteoblast.\[91\]

As mature resorptive cells, osteoclasts degrade both the organic and inorganic components of bone. When the osteoclast comes in contact with bone it polarizes, forming a “ruffled” border near the bone. This ruffle is the resportive organelle and occurs as the result of acidic vesicles being transported on microtubules to the plasma membrane where they are inserted in a process similar to exocytosis. Podosomes extend from the plasma membrane to form a ring around the ruffled border that forms a seal between the osteoclast and the bone. This ring contains lots of filamentous actin (F-actin) and proteins that link the cytoskeleton to integrins that recognize the bone matrix.\[89\] In vitro, osteoclasts are not capable of forming functional actin rings on plastic surfaces.\[93\]
The inorganic phase of bone is dissolved prior to the degradation of the bone matrix.\cite{89} Active osteoclasts secrete hydrochloric acid that resorbs the hydroxyapatite in bone. Osteoclasts also secrete proteases, such as cathepsin K, that degrade collagen and other proteins in the bone matrix.\cite{93} On the bone, osteoclasts excavate bone to form resorptive lacunae which appear as “pits.” First, the extracellular microenivonment around the bone is acidified using vacuolar H\(^+\)-adenosine triphosphatase (H\(^+\)-ATPase) in the ruffled membrane. A chloride channel in the ruffled membrane results in HCl being present in the microenvironment, which decreases the pH to approximately 4.5. The bone mineral becomes mobilized and a lysosomal protease called cathepsin K demineralizes the organic component of bone. The osteoclast endocytoses the by-products of bone degradation. The osteoclast will then detach and move to a new site of bone resorption. Termination of osteoclast activity may result from membrane receptors that sense high calcium levels or stimulation of osteoclast apoptosis.\cite{89}

**Osteoblasts**

Osteoblasts are derived from multipotent mesenchymal stem cells. Osteoblast precursors reach bone through the migration of progenitor cells from nearby connective tissues. Growth factors and cytokines that are produced in the bone marrow microenvironment are responsible for the differentiation of osteoblasts from their precursor cells. Differentiation is also affected by adhesion molecules and several systemic hormones.\cite{90} RANK-L is expressed on the plasma membranes of mature osteoblasts and lining osteocytes.\cite{91} In addition, osteoblasts produce M-CSF.\cite{93}

Mature osteoblasts appear round or cuboidal.\cite{93} Osteoblasts appear similar to fibroblasts in cell culture. Osteoblasts differ from fibroblasts in that they display a mineralized extracellular
matrix and express two different gene products. These two gene products encode for a transcription factor (Cbfa1) and a molecule that inhibits osteoblast function (osteocalcin). Cbfa1 is necessary for the differentiation of osteoblasts.\textsuperscript{[92]}

Osteoblasts secrete proteins that form the osteoid bone matrix, such as osteocalcin, osteonectin, the precursors for type I collagen, and glycosaminoglycans. The bone matrix will then become mineralized under the control of osteoblasts. Mineralization involves the deposition of hydroxyapatite. Osteoblasts control this process by controlling the local concentrations of calcium and phosphate. Bone mineralization occurs 8-10 mm behind the production of the bone matrix by the osteoblasts. The formation of the matrix controls the volume of bone formed, whereas the mineralization of the matrix determines the density of the bone by displacing water. Osteoblasts that are buried within lacunae of mineralized bone are known as osteocytes. Osteoblasts have a lifespan of approximately 3 months, and die by apoptosis.\textsuperscript{[90]}

During remodeling approximately half of all osteoblasts will develop into osteocytes, while the other half will undergo apoptosis. Osteoblasts can be subdivided into those that display an intercellular adhesion molecule (ICAM) and are ICAM-1-positive, and those that are ICAM-1-negative. ICAM-1-negative osteoblasts produce bone matrix proteins. ICAM-1-positive osteoblasts undergo apoptosis.\textsuperscript{[91]}

ICAM-1-positive osteoblasts are capable of adhering to monocytes tightly enough to induce the maturation of osteoclast precursors. IL-1 and TNF-\(\alpha\) are pro-inflammatory cytokines that activate NF-\(\kappa\)B and increase the production of RANK-L and ICAM-1 on osteoblasts, resulting in an increased interaction between osteoblasts and osteoclast precursors.\textsuperscript{[91]}
Osteocytes embedded in bone contain dendritic like processes that can detect small fractures and cracks in bone. Osteocytes are also in contact with osteoblasts, and are presumed to be capable of stimulating osteoblasts to differentiate and begin the process of bone remodeling. It is also suspected that osteocytes undergoing apoptosis release regulatory factors that induce osteoclasts to differentiate. It is also possible that osteocytes normally inhibit osteoclasts, so that osteocyte apoptosis would result in increased osteoclastogenesis. Osteocytes also produce sclerostin, which suppresses bone formation by osteoblasts. Osteoblast secretion of OPG suppresses the differentiation of osteoclasts. The initiation phase is associated with OPG suppression of RANK-L osteoclastogenesis.

**Hormonal Effects on Osteoclasts and Osteoblasts**

Calcitriol, or vitamin D₃, acts as a stimulator of bone resorption and osteoclast formation. The most active metabolite, 1,25-(OH)₂D₃ induces the fusion of committed osteoclast precursors. 1,25-(OH)₂ also stimulates RANK-L expression by stromal cells. Mature osteoclasts express receptors for 1,25-(OH)₂D₃. Therefore it is unknown whether 1,25-(OH)₂D₃ acts on mature osteoclasts directly or through osteoblasts. 1,25-(OH)₂D₃ induces IL-1 and IL-6 production by osteoblasts, which act to stimulate osteoclastic bone resorption. 1,25-(OH)₂D₃ also acts to enhance the osteoclastic bone resorption stimulated by PTH. In rats and mice, 1,25-(OH)₂D₃ is not required for osteoclast formation, as evidenced by normal osteoclast formation in vitamin D₃ receptor knockout mice. Spleen cells co-cultured with osteoblastic cells from mice, in the presence of 1,25-(OH)₂D₃ form numerous multinucleated cells that express an osteoclast phenotype.
Parathyroid hormone (PTH) from the parathyroid glands acts to maintain calcium homeostasis. It stimulates osteoclastic bone resorption and renal absorption of calcium. PTH\(_r\)P binds to the PTH receptor to activate cAMP and mediate the humoral hypercalcemia of malignancy. PTH and PTH\(_r\)P do not affect early osteoclast precursors, but do increase the number of committed mononuclear osteoclast progenitors and the number of mature osteoclasts. Murine osteoclasts have been found to express PTH receptors and PTH may act to induce the differentiation of osteoclast precursors in murine models. PTH and PTH\(_r\)P increase the expression of RANK-L on marrow stromal cells. The osteoblast appears to be the primary target for PTH. This is supported by the fact that osteoclasts only respond to PTH when co-cultured with osteoblasts.[103]

Calcitonin is secreted by the parafollicular cells of the thyroid gland. It acts to inhibit osteoclastic bone resorption. Committed osteoclast precursors and mature osteoclasts both express receptors for calcitonin. In osteoclasts, calcitonin stimulates adenylcyclase activity and the production of cAMP, which immobilizes the osteoclast and causes it to contract away from the surface of bone. Calcitonin downregulates the expression of the calcitonin receptor. Therefore, continuous exposure of cells to calcitonin results in a decrease in the effects of calcitonin.[103]

Estrogen suppresses the RANK-L-induced differentiation of osteoclasts. Estradiol stimulate OPG production and secretion in osteoblast cells, which express an estrogen receptor. Estrogen also downregulates osteoclastogenic cytokines, such as IL-1, IL-6, and TNF, that are produced by stromal cells.[103]

Prostaglandins simulate osteoclastic bone resorption in bone organ culture systems and osteoclast formation in murine marrow cultures. In humans, PGE\(_2\) inhibits osteoclastic bone
resorption and formation. In a co-culture of murine monocyte and rat osteoblastic (UMR 106) cells, PGE₂ inhibits osteoclast differentiation. In a co-culture of murine monocyte and bone marrow-derived stromal (ST2) cells, PGE₂ stimulates osteoclast formation and bone resorption.\textsuperscript{[103]}

Human growth hormone (hGH) is necessary for normal bone remodeling. Osteoblasts express hGH receptors and have a higher binding capacity for hGH when they are differentiated. GH stimulates osteoblasts to proliferate, synthesize insulin-like growth factor (IGF)-1, and synthesize IL-6.\textsuperscript{[104]}

**Bone Turnover During Menopause**

During the late stages of pre-menopause there is a decrease in bone formation followed by a rise in bone resorption. During menopause, the rate of bone turnover increases by 50-100%. During the late stages of menopause there is an increase in the rate of bone turnover which is associated with bone loss.\textsuperscript{[88]} Loss of sex steroids up-regulates osteoclast and osteoblast formation, and increases the number of osteoblast progenitors. This results in increased bone formation and resorption. The process of bone resorption is faster than bone formation. New bone loss is less dense than older bone. Therefore, increased remodeling can itself result in accelerated bone loss. In addition, without sex steroids the pits formed by osteoclasts are deeper than normal cavities. This occurs because estrogen promotes the apoptosis of osteoclasts, so that a loss of estrogen prolongs the lifespan of osteoclasts. Estrogen also has antiapoptotic effects of osteoblasts and osteocytes, so that a loss of estrogen leads to a decrease in the the lifespan of osteoblasts and osteocytes.\textsuperscript{[90]}
In the post-menopause period there is an uncoupling of the two phases of bone turnover, resulting in an increase in bone resorption and an accelerated rate of bone loss.\[^{88}\] Osteoporosis results when bone resorption is increased relative to bone formation.\[^{89}\] In the first 5-10 years after menopause, women rapidly lose bone due to the loss of estrogen production. This bone loss will eventually be compounded by the loss of bone due to the natural aging process.\[^{90}\]

**Bone loss in Surgical versus Natural Menopause in Humans**

Women who undergo surgical menopause display more rapid decreases in serum estradiol ($E_2$) levels, as compared to women undergoing natural menopause. Since $E_2$ receptors are found in bone cells, it is logical to hypothesize that changes in $E_2$ levels directly affect bone. Parathyroid hormone (PTH) increases with age, as bone turnover increases. In postmenopausal women, bone becomes more responsive to PTH. However, in combination with $E_2$, PTH has an anabolic effect on bone, meaning it acts to increase bone mineral density. A comparison of women who underwent natural or surgical menopause found that the only difference between the two groups was a significant decrease in PTH levels in women who underwent surgical menopause. Differences in bone turnover markers, estradiol, and testosterone were not different between the two groups.\[^{101}\]

The bone of the vertebral L2-L4 area is comprised predominantly of cancellous bone. The bone of the femoral neck is considered mixed bone, being comprised of both cancellous and cortical bone. One study examined the effects of surgical versus natural menopause on bone. Women undergoing natural menopause displayed a mean age at menopause of $49.1\pm3.9$, while women undergoing surgical menopause displayed a mean age at menopause of $38.3\pm4.7$. The vertebral (L2-L4) BMD for those who have undergone surgical menopause is significantly less
than the BMD for natural menopause in women ages 45-50. This indicates an increased loss in cancellous bone following the cessation of ovarian failure. Femoral neck BMD is significantly higher in surgical menopause from the ages of 55 to 70. This indicates a resistance of mixed bone to the effects of oophorectomy. In natural menopause women, a significant decrease in vertebral and femoral neck BMD is seen between the ages of 45-50 and 65-70. Body mass index was positively correlated with vertebral BMD immediately after menopause in natural menopause women. It also correlated with femoral neck BMD in all five year age groups from 45-70 in natural menopause women. Femoral neck BMD positively correlated with BMI only to age at menopause in surgically-induced menopause women. In natural menopause women, age and BMI account for 55% of vertebral BMD variability and 59% of femoral neck BMD variability. In surgically-induced menopause women, age at menopause and BMI account for 38% of vertebral BMD variability and 46% of femoral neck BMD variability. This suggests that the hormonal changes of the perimenopausal period may play a role in the interaction between BMI and BMD. After 60 years of age, surgically-induced menopause women display decreased incidences of osteoporosis in the vertebra. Between the ages of 45-50 and 55-70, surgically-induced menopause women display decreased incidences of osteoporosis in the femoral neck.[102]

**Estrogen and Bone in Men**

Men with mutant estrogen receptors display decreased bone mass. When treated with estrogen, these men exhibit increased bone mass. Thus estrogen derived from the peripheral aromatization of androgens affects bone in both men and women. Men who are completely insensitive to androgens, and present with increased testosterone and estrogen levels, display...
decreased bone mass. Therefore estrogen and androgen are likely to be involved in affecting the bone mass of adult men.\[90\]

Estrogen is implicated in bone health for both women and men. Circulating estrogen in the form of estrone (E\(_1\)) is metabolized either through the 2-hydroxyl pathway in which the inactive metabolites of 2-hydroxyestrone (2OHE\(_1\)) and 2-methoxyestrone (2MeOE\(_1\)) are formed, or through the 16\(\alpha\)-hydroxylation pathway in which the active metabolites of 16\(\alpha\)-hydroxyestrone (16\(\alpha\)OHE\(_1\)) and estriol (E\(_3\)) are formed. It has been shown in women that preferential metabolism through the 2-hydroxyl pathway display lower BMDs as compared to women with preferential metabolism through the 16\(\alpha\)-hydroxylation pathway. A cross-sectional study of men aged 50 years or older shows that the metabolism of estrogen may change bone mineral density (BMD) in men just as it does in women. When BMD is adjusted for both age and body mass index (BMI), it is found that men with a higher BMD in the proximal femur display higher urinary levels of the active metabolites 16\(\alpha\)OHE\(_1\) and E\(_3\). After adjusting for estradiol levels, the most significant correlation was seen between BMD and the 16\(\alpha\)OHE\(_1\) metabolite. Men displaying the lowest levels of 16\(\alpha\)OHE\(_1\) exhibited the lowest BMD in the femoral neck, total femur, trochanter, and intertrochanter. Free estradiol index (FEI), which is expressed as the molar ratio of total estradiol to sex-hormone-binding globulin (SHBG), is positively correlated to the BMD of the total hip, femoral neck, and intertrochanter. In this same group of men testosterone did not appear to have a significant impact on BMD. In both men and women, estradiol levels decline with age which suggests that age-related increases in bone loss are likely lined to decreasing estradiol levels.\[98\]

It has been shown that men displaying a mutation in the estrogen receptor-\(\alpha\) (ER-\(\alpha\)) gene, who are therefore incapable of responding to estrogen, display osteopenia, unfused epiphyses,
and increased markers of bone turnover. These phenotypes are also characteristic of men with homozygous mutations in the aromatase gene who are therefore incapable of synthesizing estrogen. In the aromatase deficient men, the phenotypic abnormalities can be treated with estrogen therapy. These cases demonstrate that estrogen is involved in the acquisition and maintenance of BMD but do not indicate the role of estrogen in bone loss. In men, aging results in substantial bone loss, although there is no male equivalent to the rapid loss of bone seen in women shortly after menopause. With aging in men, bone resorption increases as estrogen and testosterone levels fall. In one study, elderly men (age 68.4 ± 6.1 years) were first treated with an aromatase inhibitor and a gonadotropin-releasing hormone (GnRH) agonist that suppresses the endogenous production of testosterone and estrogen. At the same time, testosterone (T) and estradiol (E₂) patches were used to maintain normal circulating levels of these hormones. Three weeks later, the men were randomly divided into four groups. While continuing to use the aromatase inhibitor and the GnRH agonist, group A discontinued the use of the T and E₂ patches, group B continued using only the E₂ patch, group C continued using only the T patch, and group D continued using both patches. Three weeks after this all study medications were discontinued and all participants used a T patch for two additional weeks while the effects of the GnRH agonist waned. After the first three weeks, normal T and E₂ levels were replicated in all test subjects. By the sixth week of the study, markers of bone resorption, as indicated by levels of deoxypyridinoline (Dpd) and N-telopeptide of type I collagen (NTx), had significantly increased in group A. In group B levels of Dpd increased (but not to significant levels) while NTx levels had significantly increased. Larger increases in both Dpd and NTx were seen in group C. No changes were seen in group D. By the sixth week of the study, markers of bone formation, as indicated by levels of osteocalcin and the amino-terminal propeptide of type I procollagen
(PINP), had significantly decreased in group A while remaining unchanged in groups B and D. In group C, PINP levels decreased while osteocalcin levels remained unchanged. These results indicate that estrogen is involved in bone formation and resorption and prevents bone loss in elderly men. Statistical analysis on this data indicates that testosterone and estrogen both play a role in bone formation and resorption, with estrogen playing a more dominant role in bone resorption.\[99\]

**Bone Loss in the OVX Model**

A useful animal model should satisfy three criteria: “convenience, relevance and appropriateness.” A useful animal model of postmenopausal bone loss should be able to demonstrate “spontaneous or induced bone loss due to ovarian hormone deficiency” that mimics the bone loss seen in postmenopausal women. The ovariectomized (OVX) rat model of the postmenopausal state demonstrates bone loss and hormone deficiency, but fails to replicate the increase in fractures found in postmenopausal women. In women, the first ten years following menopause are marked by rapid bone loss due to increased bone resorption and turnover. During this period more cancellous bone is lost than cortical bone. There is also a decrease in intestinal calcium absorption during this period. The second phase of bone loss is marked by slower rate of bone loss.\[97\]

The OVX rat model can be used in rats that are either aged (12 months of age) or mature (3 months of age). The rats used in the OVX model need to be skeletally mature in order to mimic women undergoing postmenopausal bone loss. Female Wistar rats reach skeletal maturation by 12 months of age. However, by this time in the rat life span some cancellous bone has already been lost in the metaphysis of the long bones. In the Wistar rats, bone loss in the
cancellous bone of the proximal femur has been observed by two months post-OVX. Bone resorption is found to be increased relative to bone formation six months post-OVX. Since aged rats are expensive and a six month period is required to see an increase in bone turnover, mature rats have been used. Mature rats are those that are reproductively mature.\[^{97}\]

In mature Sprague Dawley rats (aged 90 days at OVX), OVX rats are found to display significantly increased weights as compared to control rats 370 days post-OVX. This occurs despite pair-feeding. The cancellous bone volume of the proximal tibia in control rats remains at approximately 30% until 270 days when the bone volume declines to about 10% between 270 and 540 days. This is associated with an increase in bone turnover. OVX rats display an initially rapid phase of cancellous bone loss between 0 and 100 days post-OVX that occurs at a rate of 0.82% per day. This is due primarily to an increase in bone turnover. Between 100 and 270 days, the cancellous bone volume of OVX rats stabilizes at 5-7%. At this point the rats are considered to be osteopenic. By 540 days the cancellous bone volume has declined to approximately 1%. This later, slower phase of bone loss is primarily caused by increased bone turnover. Since the increases in bone turnover are associated with an increase in bone loss, it follows that bone resorption exceeds bone formation. These events are consistent with the bone loss seen in women during the initial stages of estrogen deficiency following menopause. However, the slower rate of bone loss in women is associated with diminished bone formation that is not seen in the later stages of bone loss evidenced by OVX rats. It is thought that the short lifespan of rats does not allow for enough time to observe a decrease in bone formation during the slower phase of bone loss.\[^{96}\] Thus OVX can be said to result in an initially rapid phase of bone loss in which bone resorption and turnover are increased, followed by a slower phase of increased bone turnover and resorption.\[^{97}\]
At 14 days post-OVX, rats display an increase in longitudinal bone growth that has declined to control levels by day 270. This growth rate then increases again relative to control rats between 370 and 540 days. This is due to the fact that control rats display closure of the proximal tibial growth plate at 370 and 540 days, whereas the proximal tibial growth plates of OVX rats remain open. Between 14 and 100 days, osteoclast and osteoblast surface are significantly increased in OVX rats. Between 370 and 540 days osteoblast surface increases in OVX rats but remains stable in control rats. At all time points up to 540 days the mineralizing surface in OVX rats is significantly increased and bone formation rates are increased as compared to control rats. The increase in bone formation peaks during the first several months post-OVX (day 35 to 100) before declining close to control levels by day 270 and peaking again at later time points.[96]

In the aged OVX rat, bone loss is more prominent in the vertebra as compared to the femur, which is consistent with an increased loss of cancellous bone as compared to cortical bone. This phenomenon is also observed in menopausal women. In the aged OVX rat bone ovarian hormone deficiency results in increased rats of bone formation and resorption, with resorption exceeding formation. This is also observed in postmenopausal women. As bone mass in women falls below the peak bone mass achieved prior to menopause, there is a decrease in the ratio of cortical to periosteal bone area. This is mimicked in the OVX rat that is ovariectomized at 7 months of age and sacrificed 7 months post-OVX.[97]

In the mature OVX rat, bone resorption increases relative to bone formation so that bone turnover is increased in cancellous bone. In the vertebrae of these rats, trabecular number is decreased at 4 months post-OVX. However the cancellous bone loss observed in the vertebra of
OVX rats is not as pronounced as the cancellous bone loss seen in the metaphysis of the long bones.\textsuperscript{[97]}

In Sprague Dawley rats, the linear growth of the tibia occurs rapidly until approximately 170 days before declining. Between 6 and 18 months the proximal tibial epiphyseal growth cartilage thins, ossifies, displays an end to endochondral ossification, and closes. By 7 to 8 months, the longitudinal growth of the lumbar vertebrae has virtually ceased.\textsuperscript{[97]}

In the mature model, lower bone mass should not be automatically equated with bone loss. This is because in control rats a gain in bone mass is occurring. Therefore the OVX rats may be exhibiting impaired bone growth as opposed to increased bone loss. Bone loss can only be said to occur when the bone mass of OVX rats is less than the bone mass of control rats sacrificed at the time of OVX.\textsuperscript{[97]}

Also, rats display bone multicellular units (BMUs) that are similar to those found in adult human cancellous bone and undergo similar phases of activation, resorption, and formation. However, cancellous bone in rats must be identified as either primary spongiosa or secondary spongiosa to avoid confusing bone growth and bone remodeling activities.\textsuperscript{[97]}

Experiments on 8 month old Wistar female rats also demonstrate the bone loss observed in ovariectomized (OVX) rats. Six months post-operation, the bone strength of the femoral diaphysis of OVX rats is significantly decreased compared to rats in the sham group. In addition, the bone mineral density (BMD) of the femoral proximal and distal metaphyses, the lumbar spine, the tibial proximal metaphysis, and the tibial diaphysis is significantly lower in OVX rats at this timepoint. During the six month post-operation period the BMD of the lumbar spine and the tibial proximal metaphysis are always lower in OVX rats. Therefore, the bone mass of trabecular bone decreases immediately post-OVX, whereas the bone mass of cortical
bone does not immediately decrease. In 6 week old Sprague Dawley female rats, the OVX rats exhibit significantly higher body weight gains, food intakes, and food efficiencies over a two month period. OVX and sham rats fed a diet low in calcium display significantly decreased lumbar spine and tibial proximal metaphysis BMD. The BMDs of these areas were lower in the OVX group as compared to the sham group. When these rats are subsequently fed a diet high in calcium for 28 days the BMD of the lumbar spine and the tibial proximal metaphysis increase significantly. During the period of low calcium intake the BMD of the tibial diaphysis decreases slightly in both groups, but increases significantly during the period of high calcium intake. Both groups display similar breaking forces at the center of the femoral diaphysis. Therefore growing rats display decreased trabecular, but not cortical, bone BMD post-OVX. In addition, calcium deficiency also decreases the BMD of trabecular bone in growing rats.[94]

Another experiment examined bone loss in ovariectomized Sprague Dawley rats. Twelve week old Sprague Dawley rats that are ovariectomized and given free access to food display significantly increased body weights four weeks post-OVX. OVX rats display significantly decreased cancellous bone BMD as compared to sham-operated rats. The bone mineral content (BMC) of tibial metaphysis (cancellous bone) is significantly reduced beginning at 4 weeks post-OVX and continuing through 18 weeks post-OVX. However the cross-sectional area of the bone is not altered. Areas comprised mainly of cortical bone did not display changes as a result of OVX over the 18 week period. Also, biomarkers of bone formation (alkaline phosphatase) are reduced immediately post-OVX but are elevated by 18 weeks post-OVX. Biomarkers of bone resorption (urinary deoxypyridinoline) are significantly higher in OVX rats over the entire 18 week period. This suggests that bone resorption increases earlier than the increase in bone formation. Therefore bone remodeling is uncoupled immediately post-OVX, which could
explain the increased loss of cancellous bone in OVX rats. The bone strength in torsion and bending of the tibial metaphysis decreases significantly 4 weeks post-OVX and remains reduced up to 18 weeks post-OVX. The femoral diaphysis displays no change in maximal force, break force, or deformation post-OVX as compared to sham-operated rats.[95]

Comparing Bone Loss Between VCD and OVX mice

A single report from the laboratory of our collaborator, Dr. Patricia Hoyer, has examined the post-ovarian failure bone loss that occurs in the VCD-treated rodent as compared to the bone loss that occurs in an ovariectomized rodent; this work was conducted in C57BL/6Hsd mice. Immature (28 days old) female C57BL/6Hsd mice dosed with VCD (160 mg/kg/d, ip, 15 d) exhibit no change in the bone microarchitecture of the vertebrae, distal femur, or femoral midshaft as compared to controls pre-ovarian failure. Ovarian failure in VCD treated mice occurs approximately 48.1±2.7 days post-VCD administration. VCD treated mice display a reduced spine bone mineral density (SpBMD) 2.9 months post-ovarian failure. The SpBMD of VCD treated mice differs from the SpBMD of control mice at 3 months post-ovarian failure, but at no other time point. Ovariectomized (OVX) female mice exhibit decreases SpBMD values beginning at 1 month post-ovarian failure. OVX mice therefore display significantly lower SpBMD values as compared to controls until 6 months of age when control mice begin to lose bone mass. OVX and VCD treated mice exhibit decreased vertebral trabecular bone volume fraction, trabecular number, and connectivity density 5.3 months post-ovarian failure as compared to age-matched controls. They also both exhibit increased trabecular separation. Only OVX mice display decreased trabecular numbers and increased trabecular separation in the distal femur as compared to controls. The trabecular separation in the vertebrae and distal femur is
increased in OVX mice as compared to VCD treated mice. In the femoral midshaft, OVX mice display decreased cortical bone area, bone area fraction, and bone thickness as compared to both VCD treated and control mice. Older (approx. 8.5 mo.) VCD treated and control mice both exhibit greater mid-femoral size, cortical bone area, and cortical bone thickness as compared to young mice (3 mo.). Androstenedione levels of OVX mice are significantly lower than both VCD treated and control mice 2 months post-ovarian failure. Three weeks post-ovarian failure OVX and VCD treated mice display circulating 17β-estradiol levels that are 50% of control levels. Uterine weights at 8.5 months post-ovarian failure were decreased in OVX compared to VCD mice, as well as OVX and VCD compared to controls. Only VCD treated mice display decreased ovarian weights as compared to controls. This data suggests that VCD does not directly affect bone microarchitecture, rather the observed bone loss is a result of ovarian failure. Also, OVX mice exhibit a faster decline in SpBMD and a greater decrease in trabecular bone microarchitecture compared to VCD treated mice.\textsuperscript{70}
METHODOLOGY

In vivo experiments with Sprague Dawley rats

All experiments involving animals were approved by the University of Arizona Institutional Animal Care and Use Committee (IACUC).

Skeletally immature (one month old) and skeletally mature (three month old) female Sprague Dawley (SD) rats were obtained from Harlan Laboratories. They were housed in plastic cages and exposed to 12 hour cycles of light and dark. The rats were allowed access to food and water ad libidum. The rats were allowed to acclimate to their housing for 7 days before they were randomly distributed into one of four experimental groups. Skeletally immature (“young”) and skeletally mature (“mature”) SD female rats were administered VCD (160 mg/kg, ip; Sigma) or vehicle (DMSO, 1.25 μL/g, ip; Sigma) for 25 days. Group A consisted of young rats that were administered vehicle (dimethyl sulfoxide; DMSO; n=5). Group B consisted of young rats that were administered VCD (n=5). Group C consisted of mature rats that were administered vehicle (n=3). Group D consisted of mature rats that were administered VCD (n=2). Body weights were determined prior to each injection. Sacrifice occurred at day 36 or 38 relative to the onset of treatment, for the skeletally mature and skeletally immature rats respectively. Organ weights were determined at the time of harvest. Blood samples were obtained and saved for future analysis. Ovaries were fixed in Bouin’s fixative and stored at room temperature for future histologic analysis of ovarian follicles. The Bouin’s fixative consisted of 15 mL Picric Acid (Sigma), 5 mL 37% formaldehyde (Sigma), and 1 mL glacial acetic acid. The ovaries were placed in the Bouin’s solution for 4 hours and then transferred to 70% EtOH for 24 hours. At the end of the 24 hours, the ovaries were placed in fresh 70% EtOH.
**Vaginal Cytology**

In order to determine the reproductive status of the rats, vaginal cytology was assessed for 7 days post-treatment. Using a glass pipet, a small volume of saline was inserted into the vaginal orifice and then removed. A few drops of the cell-containing saline solution was then placed on a glass microscope slide and observed under a light microscope. The vaginal smears were indicative of proestrus if round, nucleated epithelial cells were observed; estrus if large cornified cells were observed; metestrus if a combination of leukocytes, cornified, and rounded epithelial cells were observed; and diestrus if round epithelial cells and leukocytes were observed. In addition, two phases were noted in situations where vaginal cytology indicated characteristics of more than one phase of the estrous cycle (as would exist during the transition between two phases). In some cases too few cells were apparent on the slide and a proper classification could not be observed.

**Ovarian Follicle Counts**

In research previously conducted in the lab, 3 month old female Sprague Dawley rats (Harlan Laboratories) were administered VCD (160 mg/kg b.w., ip; Sigma) or vehicle (DMSO, 1.25 μL/g, ip; Sigma) for 20-25 days. The animals were harvested on day 22, 29, 30, or 261. Some animals were sacrificed immediately post-treatment (days 22, 29, 30 relative to the onset of dosing). Other rats were sacrificed approximately 8 months post-treatment, on day 261 relative to the onset of dosing. After harvest, one ovary from each of these animals was extracted, fixed, embedded in paraffin, serially sectioned into slices, mounted onto slides, and stained with hematoxylin and eosin. A light microscope was used to determine the ovarian follicle counts for each ovary. These follicle counts were then divided by the number of ovarian
sections counted in order to obtain follicle counts per section. The ovarian follicles were identified as primordial if they displayed a single layer of squamous granulosa cells surrounding the oocyte. The ovarian follicles were identified as small primary if they displayed a single layer of round granulosa cells numbering less than 20 surrounding the oocyte. The ovarian follicles were identified as large primary if they displayed a single layer of round granulosa cells numbering more than 20 surrounding the oocyte. The ovarian follicles were identified as secondary if they displayed two layers of round granulosa cells surrounding the oocyte. The ovarian follicles were identified as antral if they displayed a large zona pellucida and multiple layers of round granulosa cells surrounding the oocyte.

**Bone Mineral Density**

BMD was obtained at baseline and two days prior to harvest using dual energy x-ray absorptiometry (DXA; PIXImus). Rats were anesthetized with Rabbit Mix and then properly aligned on the DXA machine so that their hind limbs would appear within the frame of the x-ray (Picture 1.1). Using the Piximus software, different bone areas of interest were highlighted and the bone densities of these areas were determined (Picture 1.2). The densities of the total femur, the distal 25% of the femur, the total tibia, and the proximal 25% of the tibia were determined. All absolute values of bone mineral density are expressed as gm/cm$^2$. 
Histomorphometry

The skeletally immature rats were dosed with calcein (25 mg/mL; 1μL/g, ip) on day -10 and -3 relative to sacrifice so that bone accretion rates could be determined (fig. 1.1). Primary and secondary histomorphometry indices for the proximal tibias of vehicle and VCD-treated rats (n=4/grp) were analyzed by the Center for Metabolic Bone Diseases at the University of Alabama at Birmingham.

Fig. 1.22  Image of calcein double labeling under a fluorescence microscope. Two doses of calcein were administered to mice 8 days apart. On day 1, only one calcein label appears. After the injection of the second calcein dose, two calcein labels can be visualized. The calcein flurochrome is incorporated into newly forming bone
matrix mineralization. Therefore, the distance between the two calcein labels is representative of the amount of bone formed during the 8 day period.\(^{[92]}\)

**Osteoclastogenesis with RAW 264.7 cells**

To determine the best plating conditions for developing osteoclasts from RAW 264.7 cells, a murine macrophage cell line that differentiates and fuses into multinucleated osteoclasts following exposure to RANK-L, different cell concentrations were plated with 50 ng/mL of RANK-L (Sigma) for varying periods of time. RAW264.7 cells were plated at concentrations of \(1 \times 10^3\), \(5 \times 10^3\), \(1 \times 10^4\), or \(2 \times 10^4\) cells/well, in 24-well plates. The cells were harvested on day 5, 6, or 7 of growth. Four wells were plated for each treatment group. On day 1, RAW 264.7 cells were plated in 500 \(\mu\)L of media. On day 2 of growth, all of the media in the wells was removed using a glass pipet under vacuum. Next, 700 \(\mu\)L of RANK-L containing media were slowly added down the side of the wells. Media was refreshed on day 5, by pipeting off all of the media and slowly adding 700 \(\mu\)L of fresh RANK-L containing media down the side of the wells. Any air bubbles in the wells were removed by bursting them with a 30 g needle.

RAW 264.7 cells were grown in 75 cm\(^2\) vented cell culture flasks (BD Falcon), in media containing Dulbecco’s modified eagle medium (DMEM; Cellgro), 10% fetal bovine serum (FBS; Atlanta Biologicals), and 1% penicillin-streptomycin (P/S; Invitrogen). In order to culture RAW 264.7 cells, the media in which the cells were to be plated was first brought to room temperature in a 37\(^\circ\) C water bath. Once at room temperature, the media bottle was wiped clean with 70% ethanol and placed in a sterile tissue culture hood. Using a glass pipet connected to a vacuum, the “old” media was removed from the 75 cm\(^2\) vented flask containing the RAW 264.7 cells. Five milliliters of fresh, room-temperature media was added to the 75 cm\(^2\) vented flask. A plastic squeegee was used to scrape the RAW 264.7 cells off of the bottom of the flask. One mL
of this RAW 264.7 cell-containing media was pipeted into a 15 mL tube. Four mL of fresh media were added to the 15 mL tube, for a total volume of 5 mL. This created a 1:5 dilution, and is henceforth known as the cell solution. 10 μL of the cell solution was inserted into a hemocytometer. The cells in all four quadrants of the hemocytometer were counted. The total number of cells from all four quadrants were added together and divided by four. This number was multiplied by $10^4$, in order to determine the total number of cells per mL present in the cell solution. Because of the 1:5 dilution, this number was multiplied by 5.

To determine the total number of cells required to plate RAW 264.7 cells at a particular concentration, the total number of wells to be used was multiplied by the desired concentration of cells per well. Since each well was to be plated in a total volume of 500 μL, the total number of wells to be used was multiplied by 500 μL per well. This yielded the total mL of solution that would be used. The total number of cells required to plate RAW 264.7 cells at a particular density was divided by the number of cells per mL present in the cell solution. This yielded the mL of cell solution that would be used. Subtracting the mL of cell solution to be used from the total mL of solution to be made yields the mL of fresh media that must be added to the mL of cell solution. The proper amount of cell solution and media were combined in a 50 mL tube. Each well of a 24-well plate received 500 μL. The cell solution was frequently swirled as it was pipeted into the wells, in order to maintain homogeneity. The plates were labeled and incubated at 37° Celsius in 5% CO₂.

All cells were harvested on day 5, 6, or 7. The media in the wells was removed using a pipet attached to a vacuum. Each cell was rinsed with 500 μL of 1X phosphate buffered saline (PBS; Fisher), which was then aspirated. To fix the cells to the plates, 500 μL of a 1:1 mixture
of acetone:ethanol was added to the wells for one minute. This mixture was then aspirated off. The wells were allowed to dry for approximately 10 minutes.

A tartrate-resistant acid phosphatase (TRAP) stain kit (Sigma) was used to identify TRAP-positive cells that would indicate the presence of multinuclear cells. The presence of multiple nuclei is a phenotype that is specific to osteoclasts, so that TRAP-positive cells are indicative of the presence of osteoclasts. The following protocol was used for each 24-well plate. First, 11.25 mL of deionized water (ddH₂O) was pipeted into a 15 mL conical tube. This tube was then placed into a water bath at 37° Celsius. In a sterile 15 mL conical tube, 125 mL of Fast Garnet GBC Base solution was added to 125 mL of Sodium Nitrite solution. This mixture, hereafter known as the Diazotized Fast Garnet GBC solution, was allowed to sit for at least two minutes. Once the 15 mL tube containing the ddH₂O was heated to 37° Celsius, it was removed from the water bath. To this ddH₂O was added 250 μL of the Diazotized Fast Garnet GBC solution, 125 μL of Napthol AS-B1 Phosphate solution, 500 μL of Acetate solution, and 250 μL of Tartrate solution. The 15 mL tube, now containing the TRAP solution, was then placed back into the water bath to regain any heat lost. When the 15 mL tube was re-heated to 37° Celsius, 500 μL of TRAP solution was added to each well in the 24-well plate. The plate was then incubated at 37° Celsius, in 5% CO₂ for two hours. After two hours, the TRAP stain was aspirated. Each well was rinsed with ddH₂O, which was subsequently aspirated. The plate was allowed to dry for approximately ten minutes. When dry, 300 μL of 0.1% Methyl Green (Sigma) was added in order to counter stain the wells. The Methyl Green was left in the wells for 5 minutes, before being aspirated. The wells were then rinsed with ddH₂O and allowed to dry.

To count the osteoclasts in the wells, six lines were drawn in marker on the bottom of each well, creating 16 quadrants in each well. The number of cells that appeared TRAP positive
and contained greater than three nuclei were counted as osteoclasts. The number of osteoclasts per well were counted using a light microscope. The average number of osteoclasts for each plating condition was then determined.

In order to determine whether RAW 264.7 cells could be viably co-cultured with VCD, RAW 264.7 cells were plated in 24-well plates at a concentration of 1x10^4 cells/well. VCD (0.1, 1, 10, 100, or 300 μM) was initially dissolved in DMSO so that the final concentration of DMSO present in the wells was approximately 0.001%, 0.01%, 0.13%, 1.29%, and 3.86% for the VCD concentrations of 0.1, 1, 10, 100, and 300 μM respectively. One column of four wells was not treated with VCD and served as a control. Cells were plated on day 1 in 500 μL of media. On day 2, all of the media in the wells was removed using a glass pipet under vacuum. Next, 500 μL of VCD was slowly added down the side of the wells. On day 5, the media was refreshed by pipeting off all of the “old” media and slowly adding 500 μL of VCD containing media down the side. Any air bubbles in the wells were removed by bursting them with a 30 g needle.

In order to determine whether VCD was toxic to RAW 264.7 cells, an MTT cell proliferation assay (Promega) was performed at day 5 of growth for wells containing 0, 0.1, 1, 10, 100, and 300 μM of VCD. First the MTT kit and media containing DMEM, 10% FBS, and 1% P/S were removed from the refrigerator and brought to room temperature. 200 μL of media were removed from each well of the 24-well plate and transferred to a sterile 24-well plate. Into each well of the new plate, 20 μL of MTT reagent was added, for a total volume of 520 μL. This plate was incubated at 37°C Celsius in 5% CO2 for two hours, until a purple precipitate was formed. 200 μL of detergent reagent was added to each well. The plate was left in a dark drawer for 2 hours. 200 μL from each well of the 24-well plate was then transferred to a sterile 96-well
plate. The 96-well plate was read on a plate reader at an absorbance of 570 nm. The absorbance readings that were obtained were averaged and then expressed as percent of control.

To determine the effects of VCD co-culture on RAW 264.7 cells, RAW 264.7 cells were plated in 24-well plates at a concentration of $1 \times 10^4$ cells/well. VCD (0.1, 1, 3, 10, and 30 $\mu$M) was initially dissolved in DMSO so that the final concentration of DMSO present in the wells was approximately 0.001%, 0.01%, 0.03%, 0.13%, and 0.39% for the VCD concentrations of 0.1, 1, 3, 10, and 30 $\mu$M respectively. One column of four wells was not treated with VCD and served as a control. Cells were plated on day 1 in 500 $\mu$L of media. On day 2, all of the media in the wells was removed using a glass pipet under vacuum. Next, 350 $\mu$L of 2X RANK-L containing media and 350 $\mu$L of 2X VCD were slowly added down the side of the wells. Media was refreshed on day 5, by pipeting off all of the media and slowly adding 350 $\mu$L of 2X RANK-L containing media and 350 $\mu$L of 2X VCD down the side of the wells. Any air bubbles in the wells were removed by bursting them with a 30 g needle.

**Primary cell Osteoclastogenesis**

All experiments involving animals were approved by the University of Arizona Institutional Animal Care and Use Committee (IACUC).

In order to determine the best plating conditions for growing primary cell osteoclasts from mixed bone cell cultures, bone marrow cells isolated from the tibias of 3 month old female Sprague Dawley rats (Harlan Laboratories) were plated at a concentration of $2 \times 10^5$ cells/well in a 24-well plate. Four control wells contained only osteoclasts. The other five columns were plated as follows: M-CSF alone; RANK-L alone; M-CSF and RANK-L; M-CSF, RANK-L, and PTHrP; and PTHrP alone. M-CSF (R&D Systems) was used at a concentration of 50 ng/mL.
RANK-L (R&D Systems) was used at a concentration of 100 ng/mL. PTHrP (Bachem) was used at a concentration of 100 ng/mL.

Cells were plated in 24-well plates, with either four or six wells per experimental group. Cells were co-cultured with VCD at concentrations of 1, 3, 10, or 30. The VCD was initially dissolved in DMSO, so that the final concentration of DMSO present in the wells was approximately 0.01%, 0.04%, 0.13%, and 0.39% for the VCD concentrations of 1, 3, 10, and 30 μM respectively. Cells were treated with RANK-L at a concentration of 100 ng/mL and M-CSF at a concentration of 50 ng/mL. The media used contained DMEM, 15% FBS, and 1% P/S. Any air bubbles in the wells were removed by bursting them with a 30 g needle.

Bone marrow cells were obtained from 3 month old Sprague Dawley rats (Harlan Laboratories). They were housed in plastic cages and exposed to 12 hour cycles of light and dark. The rats were allowed access to food and water *ad libidum*. After 7 days in housing, the rats were sacrificed and their tibias removed. The animals were first completely anesthetized using Rabbit Mix (1mL/kg, ip), consisting of 5mL Ketamine, 8mL 20mg/ml Xylazine, and 2mL Acepromazine. Under aseptic techniques, the skin of the lower abdominal cavity was cut open. A butterfly needle was inserted into the abdominal aorta, and blood was collected in a vacuum blood collection tube until the heart stopped beating. The skin and muscle around the hind leg was then removed. The femur was disconnected from the acetabulum and the tibia was cut directly above the paw. The tibia was placed in aluminum foil and transferred to a sterile tissue culture hood.

Using sterile scissors, both ends of the tibia were cut until the bone marrow could be observed. At all times the tibia was held with sterile hemostat clamps. The bone marrow cavity was flushed with 2 mL of media containing DMEM, PBS, and P/S using a 21 g needle attached
to a 1 mL syringe. The needle was first filled with media. The needle was then inserted into one end of the marrow cavity and was flushed slowly into a Petri dish. The contents of the Petri dish were then drawn back into the needle and the process was repeated, using the same end of the tibia. This was repeated again for a total of three flushings of one side of the tibia. The tibia was then inverted so that the needle could be inserted into the other end of the tibia. This end of the tibia was flushed twice. The final contents of the Petri dish were passed through a sterile metal screen into a clean Petri dish. This solution was then pipeted into a 50 mL conical tube.

To count the cells obtained from the bone marrow flushing, 200 μL were placed in a 1 mL tube. 800 μL of 3% acetic acid was added to lyse any erythrocytes. This created a 1:5 dilution. 10 μL of the acetic acid/cell solution was injected onto a hemocytometer with a glass cover. The cells were counted under a light microscope, in all four quadrants of the hemocytometer. The calculations used to identify the volume of cell solution and the volume of media required to yield the desired concentration of cells per well were identical to those used in the calculations for the RAW 264.7 cells. A volume of 175 μL per well was desired for the primary osteoclasts and the desired cell concentration was 2x10^5 cells/well.

Four experimental groups were used in the primary osteoclastogenesis experiments. One column served as controls, and was plated with 200 μL of cells at 2x10^5 cells/well , 100 μL of 4X M-CSF, and 100 μL of 4X RANK-L. The experimental columns were plated with 100 μL of cells at 2x10^5 cells/well, 100 μL of 4X VCD, 100 μL of 4X M-CSF, and 100 μL of 4X RANK-L. VCD was added in concentrations of 1, 3, 10, or 30 μM.

On day 4 of cell growth (with the initial plating occurring on day 1), the media in the wells was refreshed. Being careful not to disturb the cells, 200 μL of solution was pipeted off of the top of the wells. The control wells were refilled with 100 μL of media, 50 μL of 4X RANK-
L, and 50 μL of M-CSF. The experimental wells were refilled with 50 μL of media, 50 μL of 4X VCD (in 1, 3, or 10 μM concentrations), 50 μL of 4X RANK-L, and 50 μL of 4X M-CSF. Any air bubbles in the wells were removed by bursting them with a 30 g needle.

All cells were harvested on day 7. The media in the wells was removed using a pipet attached to a vacuum. Each cell was rinsed with 500 μL of 1X PBS, which was then aspirated. To fix the cells to the plates, 500 μL of a 1:1 mixture of acetone: ethanol was added to the wells for one minute. This mixture was then aspirated off. The wells were allowed to dry for approximately 10 minutes. The wells were TRAP stained and counter stained with Methyl Green, as described above.

To count the osteoclasts in the wells, six lines were drawn in marker on the bottom of each well, creating 16 quadrants in each well. The number of cells that appeared TRAP positive and contained greater than three nuclei were counted as osteoclasts.

**Statistics**

All data was expressed as mean ± the standard error of the mean. When necessary, a one-way analysis of variance (ANOVA) was used to compare the means of multiple sample groups. All analyses were performed with InStat software (2.01; Graphpad, San Diego, CA). Differences were considered significant if p<0.05 (indicated as “*” on graphs), very significant if p<0.01 (indicated as “**” on graphs), and extremely significant if p<0.001 (indicated as “***” on graphs). When the standard error of the mean for one data set is zero, this is indicated as “****” on graphs.
RESULTS

Acute In Vivo Effects of VCD on Ovarian Function in Sprague Dawley rats

Follicle Counts – Acute and Delayed Effects

Immediately post-treatment, VCD (160 mg/kg b.w.; ip; 20-25 days) administered to mature (3 months old) Sprague Dawley rats significantly (p<0.001) reduced the numbers of primordial, small primary, large primary, and secondary follicles as compared to control (figure 2.1). The numbers of antral follicles were not reduced and remained similar to the counts of antral follicles for vehicle control rats. In comparison, 8 months post-treatment, VCD significantly (p<0.001) reduced the numbers of primordial follicles, while reducing the numbers of small primary, large primary, secondary, and antral follicle types to zero (fig. 2.2). This delayed effect is consistent with a depletion of the stores of immature follicles, resulting in a lack of follicles necessary to supply the progression of folliculogenesis. This indicates that VCD is ovotoxic in Sprague Dawley rats and demonstrates that progression to ovarian failure occurs at a later time point as a result of the acute effects of VCD on ovarian follicles. It is interesting to note that fewer immature follicles are present in the ovaries of control animals as they age, which is indicative of a finite pool of primordial follicles undergoing continuous progression through folliculogenesis.

Cyclicity

Vaginal cytology during the 7 days immediately following VCD administration determined that no animal from any control or experimental group had entered persistent estrus (fig. 2.3).
Not all stages of the estrous cycle were evident in each rat. The progression through the four day estrous cycle follows a sequence of proestrus, estrus, metestrus, and diestrus. However, the cell types evident in vaginal smears may not be readily identifiable as a particular stage and cell types may not be immediately shed from the vaginal cavity, meaning that the estrous cycle stages identified using vaginal cytology may not be completely correct. As long as a transition between sequential stages of the estrous cycle is evident, the rat will not be considered reproductively senescent. In the young vehicle control and VCD-treated rats, the sequential transition between proestrus, estrus, metestrus, and diestrus was not readily apparent in any one rat. However, the estrous cycle stages through which the young rats progressed did not occur out of sequence. For example, one vehicle control rat sequentially displayed two days of diestrus, one day of estrus, one day of metestrus, and one day of diestrus over the course of five days. In the mature vehicle control rats, periods of proestrus, estrus, metestrus, and diestrus were evident in the rats, with no one rat displaying all four stages of the estrous cycle or persistent estrus. In the mature VCD-treated rats, each rat displayed two days of diestrus and one day where too few cells were apparent in the vaginal smear to determine which particular estrous stage the rat had entered. There is insufficient data to indicate that irregular estrous cycle lengths are occurring in these rats; however, this data does indicate that cessation of estrous cycles has not yet occurred.

Therefore, no animal had become reproductively senescent or anovulatory at the time of harvest and all animals displayed regular estrous cycles. This is consistent with the previous finding that ovarian follicle stores are not completely depleted as a result of the acute effects of VCD administration.

_Uterine Weights_
Uterine weight is often used as an assay of estrogen production, because decreased estrogen levels yield decreased uterotropie effects. In both young (fig. 2.4) and mature (fig. 2.5) rats, VCD treatment resulted in no acute effects on uterine weights. This data is consistent with prior results indicating normal ovarian function.

**Acute Toxic Effects of VCD in Sprague Dawley rats**

*Mortality*

Kaplan-Meier survival curves indicate that both young (fig. 2.6) and mature (fig. 2.7) vehicle control rats survived until the end of treatment. One death that occurred in the mature vehicle control group was the result of an injection error and was therefore not attributable to treatment. The young VCD treated rats also survived until the end of treatment (fig. 2.6). In contrast, the mature VCD treated rats displayed a high mortality rate beginning on day 15 of treatment. By the end of the VCD injection period, only 20% of the VCD-treated mature rats had survived (fig. 2.7). This compromises the significance obtained when comparing the mature VCD-treated group to other groups because two subjects cannot be used to find significant differences between groups of data; nevertheless trends between data sets can still be observed.

*Total Body Weight*

The average total body weights of the young vehicle control (fig. 2.8), young VCD-treated (fig. 2.9), mature vehicle control (fig. 2.10) and mature VCD-treated rats (fig. 2.11) were recorded daily over the time course of the experiment (figs. 2.8 - 2.11).

At baseline, by chance, the total body weights of the DMSO and VCD-treated young rats differed significantly (p<0.05), with the VCD rats displaying greater total body weights (fig.
This is due to naturally occurring variability in weights between individual rats. The total body weights of all both the young vehicle control and the young VCD-treated rats significantly increased (p<0.001) over the time course of the experiment, consistent with the fact that these animals were growing and skeletally immature at the onset of treatment (fig. 2.12). However, immediately post-treatment, the young VCD-treated rats exhibited significantly lower (p<0.001) total body weights at harvest as compared to controls (fig. 2.13).

At baseline, the average total body weights of the mature VCD-treated rats did not significantly differ from the total body weights of the DMSO-treated rats (fig. 2.14). In addition, the total body weights of the mature control and VCD-treated rats did not significantly increase over the time course of the experiment, consistent with the fact that these animals were no longer growing and were skeletally mature at the onset of treatment (fig. 2.14). The two surviving mature VCD-treated rats did not exhibit significantly different total body weights following treatment, as compared to controls (fig. 2.13). Moreover, as can be observed from comparison over the time course of the experiment of the average total body weights for mature vehicle controls (fig. 2.10) versus the mature VCD-treated rats (fig. 2.11), VCD treatment resulted in a greater fluctuation in daily weights during the injection period.

**Organ Weights**

All organ weights were expressed as a percentage of total body weight to account for naturally occurring variances in body weight between individual rats. The liver, spleen, kidneys, uterus, and ovaries of young vehicle control rats, young VCD-treated rats, mature vehicle control rats, and mature VCD-treated rats were weighed at harvest. The young VCD-treated rats displayed significantly increased liver weights (p<0.001) as compared to vehicle-treated controls.
All other organ weights in the young VCD-treated rats (fig. 2.4) were not significantly different from the organ weights of young vehicle-treated control rats. The organ weights of the mature VCD-treated rats (fig. 2.5) were not significantly different from the organ weights of mature vehicle-treated control rats. However, gross abnormalities were observed in both the young and old VCD-treated rats at the time of sacrifices. Four of the young VCD-treated rats displayed adhesions in their abdominal cavities and small intestines. One of the mature VCD-treated rats displayed extremely large intestines that were filled with food and feces. In addition, the uterus of this animal was adhered to the colon and bladder. This is in contrast to the young and mature vehicle-treated control rats who displayed no such adhesions or abnormalities.

**Acute Effects of VCD on Bone**

*Bone Mineral Density*

At baseline, before the start of treatment, bone mineral density (BMD) was not significantly different between control and experimental groups in both the young (fig. 2.15) and the mature rats (fig. 2.16).

VCD treatment had an acute detrimental effect on bone, which was most pronounced in the young rats. On the final day of treatment, the VCD-treated group of young rats displayed significantly decreased BMD in the total femur (9.25%; p<0.05), the distal femur (13.97%; p<0.01), the total tibia (13.76%; p<0.001), and the proximal tibia (17.53%; p<0.01) relative to controls. The observed bone loss was most pronounced in the trabecular/cancellous enriched proximal tibia (fig. 2.17).

Mature rats displayed less detrimental responses to VCD, as compared to young rats. On the final day of treatment, the VCD-treated group of mature rats did not display significantly
different BMD in the total or distal femur. However, the VCD-treated mature rats did display a
trend towards significantly decreased BMD in the total tibia (12.37%) and the proximal tibia
(7.83%) relative to controls. In contrast to the young rats, the observed bone loss was most
pronounced in the total tibia, which contains both cortical and trabecular/cancellous bone (fig.
2.18).

Histomorphometry

Because the young VCD-treated rats displayed the greatest bone loss immediately
following VCD administration, particularly in the trabecular-enriched proximal tibia, the tibias
of four of these animals were analyzed by histomorphometry. The metaphysis of the proximal
tibia of these rats was analyzed because this portion of the long bone is enriched in trabecular
bone, which is metabolically more active per until volume than cortical bone. Therefore
active bone turnover occurs more rapidly and can be more easily observed in this area of the long
bone. Only one of the calcein labels injected into the young rats was observable in the
metaphysis of the proximal tibia (fig. 2.19). Therefore, only static histomorphometric indices
could be analyzed.

Young VCD-treated Sprague Dawley rats demonstrated significantly decreased bone
volume to tissue volume ratios (%; BV/TV; -47.71% decrease, p<0.01) as compared to controls,
indicating that in a given volume of soft tissue in the metaphysis there is a smaller volume
occupied by trabecular bone (a rough equivalent to bone density). In addition, there is a
significant decrease in bone surface to tissue volume ratio relative to controls (mm\(^{-1}\); BS/TV;
-35.19% decrease; p<0.01), indicating that in a given volume of tissue area in the metaphysis the
surface of the metaphysis covered by trabecular bone declines with VCD treatment. Also,
trabecular thickness decreases as compared to controls (mcm; Tb.Th; -19.97% decrease; p<0.05), indicating thinner trabeculae as a result of VCD treatment. Furthermore, trabecular number decreases (mcm⁻¹; Tb.N; -35.11% decrease; p<0.01), indicating less trabeculae as a result of VCD treatment (fig. 2.20).

Young VCD-treated Sprague Dawley rats also demonstrated significantly increased bone surface-to-volume ratio (mm⁻¹; BS/BV; 24.50%; p<0.05) as compared to controls, which is indicative of an increased trabecular bone surface area within a given volume of bone in the metaphysis. Moreover, trabecular space in the proximal tibia is increased relative to controls (mcm; Tb.Sp; 79.72%; p<0.05), indicating larger spaces between trabeculae as a result of VCD treatment (fig. 2.20).

The ratio of erosion surface to bone surface (%; ES/BS) is increased, although not significantly, in VCD-treated rats as compared to controls. This is indicative of the percentage of bone that is eroded into lacunae. The ratio of quiescent surface to bone surface (%; QS/BS) remains relatively stable in VCD-treated rats as compared to controls. This is indicative of the percentage of bone that is inactive. The ratio of osteoblast surface to bone surface (%; ObS/BS) is increased, although not significantly, in VCD-treated rats relative to controls. This is indicative of the surface of bone that is covered by osteoblasts. Similarly, the ratio of osteoclast surface to bone surface (%; OcS/BS) is increased, although not significantly, in VCD-treated rats relative to controls. This is indicative of the surface of bone that is covered by osteoclasts. In addition, the number of osteoblasts as a function of osteoid surface (Ob/mm; N.Ob/OS) is increased, although not significantly, in VCD-treated rats as compared to controls. This is indicative of the number of osteoblasts present on the osteoid-covered surface. Similarly, the number of osteoclasts as a function of erosion surface (Oc/mm; N.Oc/ES) is increased, although
not significantly, in VCD-treated rats as compared to controls. This is indicative of the number of osteoclasts present on the erosion-covered surface.

The loss of trabeculae in VCD-treated bone (sections E, F, G, and H in fig. 2.21) as compared to the vehicle-treated control bone (sections A, B, D, and D in fig. 2.21) of young Sprague Dawley rats is readily apparent from stained micrographs of sections of the bone as compared side-by-side. In these micrographs, trabecular bone appears green. A visual survey of these micrographs clearly reveals that there is much less trabecular bone in VCD-treated young rats, thus providing graphic evidence of the totality of the specific histomorphometric changes indicated above.

**Direct Effects of VCD on In Vitro Osteoclastogenesis**

*Osteoclastogenesis with RAW 264.7 cells*

Since the observed bone loss occurred immediately post-VCD administration, when the rats were still cycling regularly and were responsive to estrogen, this would suggest that VCD may be exerting direct toxic effects on the bone. Therefore an osteoclastogenesis assay was performed using a mouse monocyte macrophage cell line (RAW 264.7 cells) that can be induced to differentiate into mature osteoclasts upon exposure to RANK-L. Effects of VCD on osteoclast formation in this system would provide evidence of direct effects of VCD in blocking the RANK-L signaling pathway in immature, differentiating osteoclasts.

First, it was necessary to determine the best plating conditions for developing osteoclasts from RAW 264.7 cells. The concentration of $1 \times 10^4$ cells/well harvested at day 6 of growth was found to yield an appropriate number of osteoclasts that could be feasibly analyzed using manual counting methods (fig. 2.22). In other words, it would be difficult to determine an accurate
manual count of osteoclasts if there were too many osteoclasts in one well, whereas with too few osteoclasts significant changes in osteoclast numbers could not be observed. An MTT assay indicated that RAW 264.7 cells could be viably cultured with VCD for 5 days in concentrations up to 10 \( \mu \text{M} \), but not above 100 \( \mu \text{M} \) (fig. 2.23). At VCD concentrations of 100 \( \mu \text{M} \) and above, cell proliferation is significantly inhibited. Therefore osteoclast formation, stimulated by addition of RANK-L in this tumor cell line, was examined. It was found that VCD (0.1, 1, 3, 10, and 30 \( \mu \text{M} \); 5 days) culture did not significantly change osteoclast counts relative to control (fig. 2.24).

**Primary cell Osteoclastogenesis**

Because no direct effects of VCD on osteoclast proliferation were observed, it was necessary to investigate possible indirect effects of VCD on osteoclast proliferation through its effects on osteoclast formation using mixed bone marrow cells. In a primary cell culture system, osteoclast formation was stimulated by addition of M-CSF and RANK-L. It was first necessary to determine the best plating conditions for developing osteoclasts from isolated bone marrow cells. Treatment of isolated bone marrow cells with 50 ng/mL of M-CSF and 100 ng/mL of RANK-L was found to create an appropriate environment for inducing osteoclastogenesis from primary cells (fig. 2.25). Therefore, counts of osteoclasts formed from the stimulation of bone marrow cells were examined. It was found that VCD (1, 3, 10, or 30 \( \mu \text{M} \)) significantly increased the number of osteoclasts at concentrations of 3 and 10 \( \mu \text{M} \), and was completely toxic to all bone marrow cell types at a concentration of 30 \( \mu \text{M} \) (fig. 2.26).
CONCLUSIONS

VCD appears to be ovotoxic in mature (3 month old) Sprague Dawley rats administered VCD (160 mg/kg b.w.; ip; 20-25 d). The acute effects of VCD on ovarian follicles include a significant reduction in the numbers of primordial, small primary, large primary, secondary, and antral follicles. The delayed effects of VCD injection, as viewed 8 months post-treatment, include a significant reduction in primordial follicles and a complete depletion of all small primary, large primary, secondary, and antral follicles. This delayed effect is a direct result of the acute toxicity of VCD in primordial ovarian follicles, because a diminished pool of primordial follicles will result in a complete depletion of ovarian follicles as folliculogenesis proceeds in the rat ovary. This is the exact pathology that is observed during menopause in humans and reproductive senescence in rats. Therefore, VCD administration in Sprague Dawley rats appears to accelerate the natural process of ovarian failure by artificially reducing the numbers of primordial follicles. This mimics the follicle depletion that occurs during menopause in humans.

As evidenced by the cyclicity data, both young and mature Sprague Dawley rats display normal estrous cycles immediately post-VCD administration. This is consistent with the follicle counts, which indicate that VCD acutely diminishes the numbers of ovarian follicles (including primordial follicles) but does not completely deplete the ovary of follicles. Therefore these rats would not be expected to display bone loss that results from estrogen depletion secondary to a loss of ovarian follicles. These data is further supported by the finding that uterine weights did not change as a result of VCD-treatment in either young or mature Sprague Dawley rats. A decline in uterine weights would have indicated a decline in estrogen levels; however, this effect
was not absorbed. Therefore, VCD does not acutely affect estrogen levels in the Sprague Dawley rat.

VCD appears to be acutely toxic to Sprague Dawley rats. This toxicity appears to be most pronounced in the mature rats, as evidenced by the high mortality rate that occurred during the period of VCD injection. Of the mature rats that did survive VCD treatment, none displayed changes in total body weights or organ weights. However, comparison of the daily recorded weights of mature VCD-treated rats to vehicle-treated control rats seems to suggest that VCD induces greater fluctuations in weight on a day-to-day basis. In addition, gross abnormalities were observed at harvest that appeared to be a direct result of VCD treatment. In the young rats, VCD treatment did not affect growth of the animals, as evidenced by the fact that these animals gained significant amounts of weight over the course of treatment. However, VCD treatment did significantly decrease the total body weights of these animals as compared to controls. The young VCD-treated rats also displayed significantly increased liver weights as a result of VCD administration. This is consistent with data indicating transient increases in liver weights in VCD-treated mice.\[58\] This may or may not indicate an attempt by the liver to detoxify the blood of any circulating VCD.

VCD also appears to acutely induce bone loss in the long bones of Sprague Dawley rats. This bone loss is most pronounced in the trabecular-enriched areas of the long bones of the young rats, which were skeletally immature at the onset of dosing. It is already known that young rats are more susceptible than mature rats to VCD-induced ovarian follicle loss, but it now appears that immature Sprague Dawley rats are also more susceptible than mature Sprague Dawley rats to VCD-induced trabecular bone loss in the hind limbs.\[60\] Because the young rats are still displaying normal estrous cycles and normal levels of estrogen secretion, this bone loss
can be attributed to the direct toxic effects of VCD. Static histomorphometry indices would seem to suggest that bone turnover is increased as a result of VCD treatment, as evidenced by non-significant increases in osteoblast surface to bone surface, osteoclast surface to bone surface, osteoblast numbers to osteoid surface, and osteoclast numbers to erosion surface. The significant decrease in trabecular number and trabecular thickness, accompanied by an increase in trabecular space, would appear to indicate that bone resorption has outpaced bone formation as a result of VCD treatment. This would explain the observed loss in bone mineral density in VCD-treated young and mature Sprague Dawley rats. This is further supported by other histomorphometric indices, such as the decrease in bone volume as a proportion of tissue volume. This overall process can be visually ascertained from micrographs of the tibial metaphysis, which demonstrate the loss of trabecular bone that results directly from VCD administration.

The VCD-induced increase in bone remodeling would suggest that VCD is altering the metabolic activity of the cells involved in bone resorption or formation. Osteoclast formation using RAW 264.7 cells would indicate that the effects of VCD on bone are not mediated through direct effects on osteoclasts. However, VCD does appear to stimulate osteoclast initiation and maturation, as indicated by a dose-dependent increase in osteoclast formation subsequent to VCD treatment of mixed bone marrow cells. It is unknown whether VCD is acting on osteoblasts or other cell types to increase osteoclastogenesis in vitro and in vivo.

A useful animal model should satisfy three criteria: “convenience, relevance and appropriateness.”[97] In comparison to the OVX model, the VCD model of ovarian failure does not appear to be as convenient a model for demonstrating cancellous/trabecular bone loss. Whereas the OVX model immediately enters a period of ovarian failure following treatment (ovariectomy), the VCD model does not. In addition, only bone loss occurring post-ovarian
failure in the skeletally mature Sprague Dawley rats would accurately mimic human post-
menopausal bone loss. However, not enough mature VCD-treated Sprague Dawley rats survive
treatment for this effect to be observed. Furthermore, while ovariectomy does not directly alter
bone metabolism, VCD appears to have direct toxic effects on bone.

The final conclusion of this thesis is that the deleterious effects of VCD on bone in SD
rats may compromise the utility of VCD in this animal for bone research. Bone researchers
should not use a young (1 month at start of treatment) Sprague Dawley VCD (160 mg/kg/d; i.p.;
25 d) model, because of the direct toxic effects of VCD on trabecular bone. The bone loss that
occurs in this model would be a potential confounding factor for any research involving bone
and would alter any indices of bone formation, bone resorption, bone turnover, and bone
microarchitecture. Furthermore, mature (3 months at start of treatment) Sprague Dawley VCD
(160 mg/kg/d; i.p.; 25 d) models should not be used in any research because of the idiopathic
toxicity that occurs in these animals, as evidenced by an 80% mortality rate.
Figure 2.1

APPENDIX A

Follicle Counts - Acute VCD Effects - Mature rats

Ovarian Follicle Type

DMSO
VCD

Follicle Counts

Primordial
Small Pre
Large Primary
Secondary
Antral
Figure 2.2

Follicle Counts - Delayed VCD Effects (8 months post-treatment)
- Mature rats

Ovarian Follicle Type

- Primor
- Small Prim
- Large Prim
- Secondary
- Atral

Follicle Counts

DMSO
VCD
### Cyclicity Data – Young Controls

<table>
<thead>
<tr>
<th>Animal</th>
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<th>36</th>
<th>37</th>
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<td>M</td>
<td>D</td>
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<td>P/E</td>
<td>E</td>
<td>M</td>
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<td>P/E</td>
<td>E/M</td>
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### Cyclicity Data – Young VCD

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<td>D</td>
<td>D/P</td>
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<td>D</td>
<td>P/E</td>
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<td>D</td>
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<td>E</td>
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<td>B-82</td>
<td>D</td>
<td>P</td>
<td>E</td>
<td>E</td>
<td>D</td>
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<tr>
<td>B-84</td>
<td>D</td>
<td>P</td>
<td>P/E</td>
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### Cyclicity Data – Mature Controls

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<td>C-87</td>
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<td>D</td>
<td>P</td>
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<tr>
<td>C-88</td>
<td>E</td>
<td>E</td>
<td>M/D</td>
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### Cyclicity Data – Mature VCD

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<td>Diestrus</td>
<td>D</td>
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<td>Too few cells to classify</td>
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</table>
Figure 2.4

Animal Organ Weights (g) Expressed as % of Total Body Weight (g) Day 38 Relative to Treatment - Young rats (age 79 d)
Figure 2.5

Animal Organ Weights (g) Expressed as % of Total Body Weight (g)
Day 36 Relative to Treatment - Mature rats (137 d)
Figure 2.6

Survival of Young rats: Survival proportions

Days post-start of treatment

Percent survival

- Young Vehicle Controls
- Young VCD
Figure 2.7

Survival of Mature rats: Survival proportions

Days post-start of treatment

Percent survival

- Mature Vehicle Controls
- Mature VCD
Figure 2.8

Daily Weights - Young Controls

Day (Relative to DMSO treatment)

Weight (g)
Figure 2.9

Daily Weights - Young VCD

Day (Relative to VCD treatment)

Weight (g)

B-76
B-77
B-79
B-82
B-84
Figure 2.10

**Daily Weights - Mature Controls**

- **Weight (g):** 0, 50, 100, 150, 200, 250, 300
- **Day (Relative to DMSO Treatment):** 4, 8, 12, 16, 18, 22, 26, 31, 36

- **Lines:**
  - C-87
  - C-88
  - C-89
Figure 2.11

Daily Weights - Mature VCD

Day (Relative to VCD Treatment)

Weight (g)

D-97
D-99
Figure 2.12

Total Body Weights - Young Rats

Average Total Body Weight (g)

- Baseline
- Day 36

DMSO
VCD
Figure 2.13

Post-Treatment Total Body Weights

![Bar chart showing post-treatment total body weights for DMSO and VCD groups across young and mature ages. The chart indicates a significant difference (*) between the groups.](image-url)
Figure 2.14

Total Body Weights - Mature Rats

Average Total Body Weight (g)

Baseline | Day 36

DMSO | VCD
Figure 2.15

Baseline BMD - Young rats

Absolute Values

DMSO
VCD

Bone Site

Total Femur
Distal Femur
Total Tibi
Proximal Tibia
Figure 2.16

Baseline BMD - Mature rats

Absolute Values

<table>
<thead>
<tr>
<th>Bone Site</th>
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<th>VCD</th>
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<tbody>
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<td></td>
</tr>
<tr>
<td>Distal Femur</td>
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<tr>
<td>Total Tibia</td>
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<td></td>
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<tr>
<td>Proximal Tibia</td>
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</table>
Figure 2.17

BMD - Day 36 Relative to Treatment - Young rats (age 77 d)

Bone Site

Absolute Values

DMSO
VCD

Bone Site

Total Femur
Distal Femur
Total Tibia
Proximal Tibia
Figure 2.18

BMD - Day 36 Relative to Treatment - Mature rats (age 137 d)

- **DMSO**
- **VCD**

Bone Site

- Total Femur
- Distal Femur
- Total Tibia
- Proximal Tibia
Figure 2.20

Metaphysis Trabecular Bone Histomorphometry - Static Indices

Primary data and Derived Indices
Figure 2.21

Tibial Metaphysis-Control (A-D) and VCD-treated (E-H)
Figure 2.22

Osteoclastogenesis - RAW 264.7 Cells

- Harvest Day 5
- Harvest Day 6
- Harvest Day 7

Osteoclast Counts

Cell Concentration (cells/well)
Figure 2.23

MTT Assay - Raw 264.7 - 5 days

% of control

uM VCD

0 0.1 1 10 100 300

0 20 40 60 80 100 120

*** ***
Figure 2.24

RAW 264.7 Osteoclastogenesis

Osteoclast Counts

μM VCD

0 0.1 1 3 10 30
Figure 2.25

Osteoclast Formation - Primary Cells

<table>
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<tr>
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<th>M-CSF</th>
<th>RANK-L</th>
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</tbody>
</table>
Figure 2.26

Primary Cell Osteoclastogenesis

![Bar chart showing osteoclast counts at different concentrations of VCD (μM) with statistical significance indicated by * and ****.](image)
REFERENCES


